Heme oxygenase-1 and chronic kidney disease:
a translational study of oxidative stress and atherogenesis

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Heme oxygenase-1 and chronic kidney disease:

a translational study of oxidative stress and atherogenesis

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Heme oxygenase-1 and chronic kidney disease: a translational study of oxidative stress and atherogenesis

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Table of contents

	List of abbreviations	7
Chapter 1	Introduction: The role of oxidative stress in CKD-related accelerated atherosclerosis	11
Chapter 2	Study objectives	51
Chapter 3	Atherosclerotic lesion characterization in Chronic Kidney Disease : The 'vulnerable plaque' phenotype	55
Chapter 4	Heme oxygenase-1 overexpression mitigates oxidative stress-induced apoptosis in uremic endothelial cells	85
Chapter 5	HO-1 functional promoter polymorphisms and clinical outcomes in patients with and without CKD	109
	5.1 Association of HO-1 (GT)n promoter polymorphism and cardiovascular disease: a reanalysis of the literature	111
	5.2 HO-1 genotype and cardiovascular disease in a dialysis population	137
Chapter 6	General discussion	153
Chapter 7	Future prospects	169
Chapter 8	Summary / Samenvatting	177
	Curriculum Vitae	185
	Publications	189

Abbreviations

αSMA: α-smooth muscle actin
αTOH / $\alpha TO^{\cdot}$: alpha-tocopherol or Vitamin E
8OHdG:
ADMA: asymmetric dimethyl arginine
AGEs: Advanced glycation endproducts
AHT: arterial hypertension
AMI: acute myocardial infarction
APO E: Apolipoprotein E
ARE: antioxidant response elements
AVF: arteriovenous fistula
BH4 : (6R)-5,6,7,8-tetrahydro-l-biopterin
BMI: body mass index
BVR: Biliverdin Reductase
CAD: coronary artery disease
CASP3: caspase 3
CAT: catalase
CD36: cluster of differentiation 36
CFs: Cocoa Flavonols
CKD: chronic kidney disease
CO: carbon monoxide
CoQ10: coenzyme Q_{10}
COX: cyclooxygenase
cROS: Intracellular, Cytoplasmic ROS
CVA: cerebrovascular accident
CVD: cardiovascular disease
CVRF: cardiovascular risk factors
DDAH: dimethylarginine-dimethylaminohydrolase
DMSO: dimethyl sulfoxide
EBM and EGM-2 MV: Endothelial cell basal or growth medium, microvascular
ECL: Enhanced chemiluminescence
eGFR: estimated glomerular filtration rate
eNOS: endothelial Nitric oxide synthase
ESRD: end stage renal disease
FACS: Fluorescent activated cell sorting
FCA: fibrous cap atheroma
FCP: fibrocalcific plaque
Fe^{2+}: Iron
GPx: Glutathione Peroxidase
GSH / GSSG: Glutathione (reduced form) / Glutathione (oxidized form)
$(GT)_n$: guanidine thymidine dinucleotide ([GT]n) repeat length

H&E: hematoxylin and eosin
H_2O: water
H_2O_2: hydrogen peroxide
HD: hemodialysis
HDL: high density lipoprotein
HOCl: hypchlorous acid
HO-1: heme oxygenase 1
hsCRP: high sensitive CRP
HS / US: Healthy serum / Uremic serum
HUAEC: Human Umbilical Artery Endothelial Cells
HWE: Hardy-Weinberg equilibrium
ICAM: intercellular adhesion molecule
IHC: Immunohistochemical
iNOS: inducible Nitric oxide synthase
IL: Interleukin
IS: Indoxyl sulfate
(P)IT: (pathological) intimal thickening
IV: intravenous
IX: intimal xanthoma
Keap1: Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1
LDL: low density lipoprotein
LM: Light Microscopy
Ln: logaritmus naturalis
$LOO^{\cdot}$ / LOOH: lipid peroxyl
LPox: Lipoxygenases
LT: Leukotrienes
MCP1: Monocyte chemoattractant protein 1
MDA: Malondialdehyde
Mg: Magnesium
MMP2: matrix metalloproteinase 2
MRC: Mitochondrial Respiratory Chain
MPO: Myeloperoxidase
mROS: mitochondrial reactive oxygen species
NADPH: Nicotinamide Adenosine Dinucleotide Phosphate
NO: Nitric oxide
NOX: NADPH oxidase
NQO1: NADPH Quinone oxidoreductase 1
Nrf2: nuclear factor-erythroid 2-related factor 2
NF-κB: Nuclear Factor Kappa B
O_2: Oxygen
$ONOO^{\cdot}$ / ONOOH: peroxynitrite / peroxynitrous acid

·OH: Hydroxyl
OR: Odds ratio
OxLDL: oxidized low-density lipoprotein PAD: peripheral artery disease
PBS: phosphate buffered saline
PCI: percutaneous coronary intervention
pCS: p-cresyl sulfate
PD: Peritoneal Dialysis
PDGF: platelet derived growth factor
Pen/Strep: Penicilline/Streptomycine
PMN: polymorphonuclear cells
PRMT: protein arginine N-methyltransferase
PTH: parathyroid hormone
(G)Px: (Glutathione) Peroxidase
ROS: reactive oxygen species
(V)SMC: (vascular) smooth muscle cells
SIN-1: 3-morpholino-sydnominine
SNP: single nucleotide polymorphism
SOD: superoxide dismutase
TNFα: tumor necrosis factor alpha
TXA: thromboxanes
VCAM: vascular cellular adhesion molecule
Vit.C: Vitamin C
XO: Xanthine oxidoreductase
XO: Xanthine dehydrogenase

CHAPTER 1

Introduction

The role of oxidative stress in CKD-related atherosclerosis：Contents

Characteristics of cardiovascular disease in CKD: arteriosclerosis and accelerated atherosclerosis

Oxidative stress

Is oxidative stress the culprit in CKD-related accelerated atherosclerosis?

Overview of oxidative and antioxidative mechanisms involved in atherosclerosis in the general and CKD population

A. Sources of oxidants in the vessel wall contributing to atherosclerosis
   I. NAD(P)H oxidases
   II. Endothelial nitric oxide synthase (eNOS)
   III. Xanthine Oxidoreductases
   IV. Myeloperoxidases
   V. Lipoxygenases
   VI. Mitochondrial respiration
   VII. Other forms of oxidants

B. Antioxidant Defense mechanisms in the vessel wall
   I. Nonenzymatic antioxidants
      a. Proteins
      b. Nonproteinaceous antioxidants
         - Water Soluble antioxidants
            - Ascorbate (Vitamin C)
            - Flavonoids and Polyphenols
            - Uric Acid
            - Bilirubin
            - Tempol
         - Lipid Soluble antioxidants
            - Vitamin E
            - Coenzyme Q_{10}
   II. Enzymatic antioxidants
      a. The 'Classic' antioxidative enzymes
         - Superoxide dismutase (SOD)
         - Catalase and peroxidases
         - Thiol-disulfide oxidoreductases
      b. Antioxidative response element-driven enzymes; Heme oxygenase-1

Summary

The role of oxidative stress in CKD-related atherosclerosis.

Characteristics of cardiovascular disease in CKD: arteriosclerosis and accelerated atherosclerosis

Cardiovascular disease (CVD) remains an important socio-economic health problem and the leading cause of death throughout the world. According to the American Heart Association, more than 16 million people suffer from coronary heart disease in the USA.[1] In Europe, each year CVD causes over 4 million or 47% of all deaths.[2]

Chronic Kidney Disease (CKD) is characterized by a loss of kidney function, in many cases progressively deteriorating towards end-stage renal disease with the need to renal replacement therapy. Patients are considered to have CKD when having either structural damage to the kidneys detected by imaging or the presense of proteinuria or when having a decreased glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for 3 months or more, irrespective of cause. Kidney disease severity is classified into five stages according to the level of GFR.

CKD is characterized by a high burden of cardiovascular morbidity and mortality.[3] Recent data indicate that the impact of renal insufficiency on cardiovascular disease (CVD) already begins with minor renal dysfunction. Not only does CVD appear already at a younger age as compared to the general population,[4] but its progression is fast as well and serious consequences such as myocardial infarction, cerebrovascular insults, amputation and eventually cardiovascular death are common.[5] Two distinct but overlapping processes characterize CKD-related CVD: arteriosclerosis and accelerated atherosclerosis.

So-called arteriosclerosis is considered a hallmark feature of CKD-related arterial disease. The process of arteriosclerosis contrasts with atherosclerosis because it is limited to the media and does not cause arterial lumen narrowing. Generally, arteriosclerosis is characterized by concentric media hyperplasia and vascular smooth muscle cell hypertrophy, accompanied by increased collagen formation resulting in wall thickening and subsequent calcification. This results in increased arterial stiffness,[6] which translates in increased pulse wave velocity following the cardiac systolic contraction of the heart. The clinical consequences are increased pulse pressure, microvascular end-organ damage as a consequence of higher systolic pressure injury, impaired diastolic perfusion of the vascular beds (particularly relevant to the coronary circulation), cardiac remodeling and subsequent risk of malignant arrhythmias.[7-9] Although arteriosclerosis and atherosclerosis are two distinct entities, they can occur together and a pathophysiological interplay has been described.[10]

Atherosclerosis is the major cause of CVD in the general population and shows particular characteristics in CKD patients. Translated literally from Greek,

atherosclerosis means 'pasty (athere) hardness (skleros)'. It is characterized by an accumulation of cholesterol deposits in macrophages in the arterial wall of medium and large-sized arteries: Plasma low density lipoprotein (LDL) is transported across the intact Endothelium and becomes entrapped in the ECM (Extracellular Matrix) of the subendothelial space where it is subjected to oxidative modifications to produce highly oxidized LDL (OxLDL). The oxLDL particles are recognized by macrophage scavenger receptors: Scavenger Receptor-A (SR-A), CD36 and CD68. The macrophages take up the OxLDLs, become enlarged and full of lipid. These cells accumulate in tissues and are transformed into lipid-laden foam cells, a process which eventually leads to an inflammatory response with the proliferation of monocytes, T-cells and smooth muscle cells (SMCs), leading to well-defined plaque lesions in the arterial wall. Atherogenesis has been extensively studied and several hypotheses regarding its initiation have been proposed, starting from endothelial cell injury over lipoprotein retention models to the oxidative modification hypothesis model, proposed by Steinberg et al. in 1989[11]: This hypothesis was mainly based on the concept that native LDL is not atherogenic. However, once chemically modified, LDL is rapidly internalized by macrophages by a so-called 'scavenger receptor pathway'. One mechanism by which cells do recognize LDL as a substrate for the scavenger receptor pathway is through oxidation of the LDL lipids and resulting modification of apolipoprotein B-100. The oxidative modification hypothesis poses that LDL indeed becomes entrapped in the subendothelial space, but must then be oxidatively modified by resident vascular cells, such as endothelial cells, macrophages and smooth muscle cells. Oxidized and minimally modified LDL have been shown to lead to endothelial cell dysfunction, and to induce formation of monocyte chemotactic protein-1 (MCP-1) in both smooth muscle and endothelial cells.[12] MCP-1 induces the recruitment of inflammatory cells. Macrophages accumulate and foam cell formation is initiated by lipid uptake. The subsequent inflammation may result in a continued LDL-oxidation, predisposing for a full-blown atherosclerotic process. Nowadays, it has become clear that oxidative stress is not the only initiating and injurious event in the atherosclerosis process and an 'oxidative response to inflammation model' has been proposed.[13] It is indeed generally recognized that both chronic inflammation and oxidative stress play reinforcing key roles in the initiation, propagation and development of atherosclerosis.

Atherosclerotic plaque progression is generally categorized into several stages: lesion initiation (formation of fatty streaks and reversible plaques), lesion formation to fibrous cap atheromata and eventually formation of complex plaques. The latter can evolve into so-called "vulnerable plaques", i.e. unstable and prone to complications such as rupture and thrombosis. Currently, it is widely accepted that cardiovascular events result mostly from (unstable) plaque complications rather than progressive plaque

enlargement and vessel narrowing.[14-16] Traditional risk factors of the vulnerable plaque are high total cholesterol, low HDL and high hsCRP levels.[17] The beneficial effects of common anti-atherosclerotic therapies such as statin treatment are also mainly mediated through plaque stabilization.

Oxidative stress

Oxidative stress is defined, in general, as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, potentially leading to damage. In other words, oxidative stress results from either excessive formation or insufficient removal of highly reactive oxygen or nitrogen species (for convenience both abbreviated by 'ROS') as a consequence of an overabundance of free radicals or lack of antioxidant capacity after an environmental or behavioral stress. Oxidative stress must be differentiated from oxidative damage. Indeed, even a severe oxidative assault accompanied by the depletion or loss of antioxidants must not necessarily lead to oxidative damage. In other words, ROS are not obligatory injurious. At moderate concentrations, ROS function as mediators and second messengers in cell signaling and proliferation, activation of metalloproteinases or transcription factors and apoptosis. ROS at small amounts can be adaptive whereas only in excessive or sustained concentrations they are associated with cell death through apoptosis and necrosis.[18,19]
Within the vessel wall, oxidants can be generated by cellular and extracellular mechanisms, from enzymatic as well as non-enzymatic sources (Table 1.1; extensively reviewed in Stocker et al.[13]).

Table 1.1 Free radicals and nonradical oxidants relevant to the vasculature

Free Radicals	Formula	Nonradical oxidants	Formula
Superoxide anion	$^{\cdot}O_2^-$	Hydrogen peroxide	H_2O_2
Hydroperoxide	$HOO^{\cdot}$	Peroxynitrite	$ONOO^-$
Peroxyl / alkoxyl radical	$ROO^{\cdot}$ / $RO^{\cdot}$	Peroxynitrous acid	ONOOH
Hydroxyl radical	$^{\cdot}OH$	Hypochlorous acid	HOCl
Nitrogen mono-/dioxide	$^{\cdot}NO$ / $^{\cdot}NOO$	Nitrosothiols	RSNO
Carbon centered radical	$-C^{\cdot}$		
Thiyl and perthiyl radical	$RS^{\cdot}$ / $RSS^{\cdot}$		
Transition metal ions	Fe, Cu		

Adapted from Stocker et al.

Free Radicals ('Primary' or 1e oxidants) are defined as independently existing species carrying one or more unpaired electrons. When they meet, two radicals join the unpaired electrons to a covalent bond in a kinetically fast reaction, resulting in a nonradical product. For example, superoxide anion ($^{\cdot}O_2^-$) and nitrogen monoxide ($^{\cdot}NO$) will combine to form peroxynitrite ($ONOO^-$).

$$^{\cdot}O_2^- + {}^{\cdot}NO \rightarrow ONOO^-$$

More commonly however, free radicals will attack nonradical molecules or consume a hydrogen atom from molecules with a C-H, O-H or S-H bond. Such molecules are compounds like antioxidants or cofactors, proteins, sugars, lipids or nucleic acids. The reaction gives rise to a new radical molecule and as such a chain reaction is initiated. In the process of lipid peroxidation for instance, a hydrogen atom from a fatty acid chain is taken by a free radical ($R^{\cdot}$) resulting in a carbon-centered new radical ($L^{\cdot}$) which in turn binds to O_2 with the generation of a lipid peroxyl radical ($LOO^{\cdot}$).

$$R^{\cdot} + LH \rightarrow L^{\cdot} + RH$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

This lipid peroxyl radical will then react with another lipid nearby, continuing the chain reaction. Depending on the reactivity of the radical, molecules with weaker or stronger hydrogen bonds will be attacked. α-Tocopherol (αTOH) is the major lipid-soluble antioxidant in humans, carrying a weak chromanol O-H bond: the scavenging of $LOO^{\cdot}$ which stops the lipid peroxidation chain reaction is believed to be the chemical basis for its antioxidative mechanism.

Next to free radicals, nonradical (or Secondary (2e)) products are also important for oxidative stress in CVD development. Examples are the abovementioned $ONOO^-$ or hydrogen peroxide (H_2O_2). The latter is mainly a weak oxidant and predominantly active on protein thiol (-SH) groups, heme proteins and amino acid radicals. Another mechanism of H_2O_2 mediated oxidative stress is the generation of hydroxyl radicals ($^{\cdot}OH$) through transition metal-catalyzed decomposition of H_2O_2, as in the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH.$$

The formation of hydroxyl radicals generally results in oxidative damage. Peroxynitrite is a weak oxidant, but in biological systems is readily converted to its highly reactive protonated form, peroxynitrous acid (ONOOH). An important difference between the free radicals and nonradical oxidants resides in their target molecules: non-radical oxidants have a predilection for proteins rather than lipids. More specifically cysteine (thiols) and methionine residues are the preferred targets. But tyrosine, tryptophan and lysine residues can be oxidized as well. Which amino acid will be the ultimate nonradical target is mainly determined by the relative abundance of the amino acid and the local environment. Examples of these reactions are the ONOOH-mediated oxidation

of the zinc-thiolate cluster of eNOS and the generation of 3-chloro- and 3-nitrotyrosine as typical oxidation products of hypochlorous acid (HOCl) or ONOOH respectively.
A variety of cellular enzymes give rise to ROS (1e or 2e) or other **oxidants**, including NAD(P)H oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), myeloperoxidases, arachidonic acid metabolizing enzymes (lipoxygenases, cyclooxygenase and cytochrome p-450 enzymes) and the mitochondrial respiratory chain. Non-enzymatic sources of oxidative stress include transition metals, lipid peroxyl radicals and hydroxyl radicals.
As mentioned above, oxidative damage occurs when an imbalance between oxidants and **antioxidants** in favor of the oxidants has been effectuated. This implies that not only excessive amounts of oxidants, but also deficient or failing antioxidant mechanisms can contribute to atherogenesis. Like the oxidants, antioxidants come in enzymatic and non-enzymatic, cellular and extracellular variants. Superoxide dismutase (SOD), catalase, glutathione peroxidase, transferase and reductase, thiol-disulfide oxidoreductase and peroxiredoxins constitute the classic antioxidant enzymes. These are largely cell-associated proteins involved in the maintenance of intracellular redox balance and extracellular antioxidants. Other antioxidant and anti-inflammatory enzymes are those driven by the antioxidant response elements (ARE), regulated mainly by Nrf2: NADPH quinone oxidoreductase, glutathione reductase and heme oxygenase-1. Non-enzymatic antioxidants include metal sequestrating proteins such as ferritin, albumin, haptoglobin and hemopexin, trace elements zinc, selenium, water-soluble antioxidants such as ascorbate (vitamin C), uric acid, flavonols and bilirubin and lipid soluble antioxidants such as vitamin E (tocopherol (TOH)) and Coenzyme Q_{10}.
At present, a wealth of data is available linking ROS and oxidative stress to the pathogenesis and disease progression of atherosclerosis. Not only lipid peroxidation,[20] but also smooth muscle cell proliferation, endothelial dysfunction,[21-24] metallo-proteinase activation,[25,26] tissue remodeling and plaque instability with subsequent plaque rupture have been described to result at least partially from ROS mediated processes. These findings are corroborated by clinical evidence linking increased oxidative stress parameters to increased risk of cardiovascular events.[27,28]

Is oxidative stress the culprit in CKD-related accelerated atherosclerosis?

The accelerated atherosclerosis process observed in CKD patients is at least partly related to the high prevalence of traditional CVD risk factors in this population: e.g. advanced age, hypertension, diabetes, smoking and dyslipidemia. However, there is a widely accepted consensus that also non-traditional risk factors contribute to the particularly devastating CVD of CKD. Indeed, uremia-related factors such as uremic

toxins, chronic inflammation, adipokine imbalance, coagulation disorders, protein energy wasting, endothelial dysfunction as well as volume dysregulation and bone mineral disorder (CKD-MBD) have been found to be associated with CVD progression.[29] Many authors hypothesize that increased oxidative stress is the unifying concept that initiates and feeds these processes that ultimately lead to CVD.[30] Indeed, several findings in uremic patients point to an imbalance favoring the pro-oxidative state: uremia-related as well as dialysis-related factors (Table 1.2) have been described as leading to the increase of oxidants and decrease of the antioxidative capacity.[31] Dialysis methods may induce oxidative stress through the exposure to bioincompatible (cellulosic and synthetic) membranes, as was seen in acute[32,33] and chronic hemodialysis[34] studies. Indirect evidence points towards a role for trace amounts of endotoxin (LPS) in the dialysate to trigger ROS production via the activation of polymorphonuclear leukocytes (PMN).[35,36] Furthermore, beneficial substances such as water-soluble antioxidants are lost during dialysis.[37] Uremia has also been associated with increased oxidative stress through chronic inflammation and the accumulation of uremic toxins. As an example plasma homocysteine, accumulating from the early stages of CKD onwards, may contribute to a pro-oxidant state by interacting with H_2O_2.[38] Protein-bound uremic toxins, such as p-cresyl-sulfate and indoxyl sulfate (IS),[39-42] have been described to induce oxidative stress and subsequent endothelial dysfunction. Asymmetric dimethylarginine (ADMA), a well-known eNOS inhibitor accumulates in CKD.[43] Protein energy wasting and malnutrition are characterized by hypoalbuminemia, hypovitaminosis C, deficiencies in trace elements and as a consequence result in deficiencies of nutritional antioxidants.[44,45]

Table 1.2 Uremia- and dialysis-related factors contributing to oxidative stress.

Source of Oxidants	**Uremia-related**	**Dialysis-related**
Leucocytes	Intravenous Iron infusion	Membrane incompatibility
"oxidative burst"		LPS, cytokines
NADPH oxidases	Uremic toxins: Indoxyl sulfate	Intravenous Iron infusion
	Homocysteine p-cresylsulfate	
eNOS uncoupling	ADMA	
Anti oxidant losses	**Uremia-related**	**Dialysis-related**
Vitamin C	Dietary restrictions	Increased permeability
	Loop diuretics	loss of watersoluble antioxidants
Albumin	Protein energy wasting	malnutrition
	malnutrition	
GSH/GSSG	Anti-oxidant depletion	Anti-oxidant depletion
Trace elements Se, Zn	Decreased intestinal absorption	NA

Type of oxidant or antioxidant (left column, above and below) which is disturbed by either uremia related (middle column) or additional dialysis related (right column) mechanisms. ADMA asymmetric dimethylarginine, GSH/GSSG: reduced/oxidized glutathione, LPS Lipopolysaccharide, Se Selenium, Zn Zinc. The table summarizes the abovementioned findings from the literature (references are mentioned in the text)

While a direct causal link between increased oxidative stress and accelerated atherosclerosis in CKD has not yet been unequivocally demonstrated, many groups have reported correlations between oxidative stress (markers) and clinical atherosclerosis.[46,47,48-51] In what follows an extensive overview of oxidative and antioxidative mechanisms involved in atherosclerosis is described with focus on the particular context of CKD.

Overview of oxidative and antioxidative mechanisms involved in atherosclerosis in the general and CKD population (Table1.2; Figure 1.1)

A. Sources of oxidants in the vessel wall contributing to atherosclerosis

I. NAD(P)H oxidases

NAD(P)H oxidases (NOXs) are probably the major sources of ROS in the vasculature: more specifically $^{\cdot}O_2^-$ and H_2O_2 are generated by reduction of molecular oxygen in the presence of NADPH as electron donor.[52] All five NOX enzymes consist of 2 heme containing transmembrane oxidoreductases that span the membrane six times. NOX4 is the most abundant isoform in the vessel wall and is present in multiple cell types including endothelial cells, fibroblasts and to a lesser extent vascular smooth muscle cells.[53] NOX4 has also been reported in immune cells such as macrophages.[54] A variety of stimuli can activate NOXs, including Angiotensin II, thrombin, Interleukin-1 (IL1), tumor necrosis factor α (TNFα) and platelet derived growth factor (PDGF). In contrast to phagocytes, endothelial cells exhibit low but basal activity for $^{\cdot}O_2^-$ generation, and even after activation, only modest amounts of $^{\cdot}O_2^-$ are produced, suggesting a modulating role for regulatory proteins and cell signaling rather than oxidative damage.[55,56] Increased NOX4 expression and vascular $^{\cdot}O_2^-$ generation have been described in human atherosclerotic lesions[53] and in *in vitro* models, among others. in response to oxidized lipids including oxLDL and oscillatory shear stress.[57] The precise role of NOX4 in atherosclerosis remains however to be determined. There are no data on the influence of uremia on vascular NOX4 expression and atherosclerosis in humans. There is however a considerable amount of evidence for an increased NOX4 activity and higher $^{\cdot}O_2^-$ levels in uremia. Hemodialysis has been shown to induce NOX4 expression in neutrophils of dialysis patients, by inducing the so-called respiratory burst.[31,35] *In vitro* data show Indoxyl sulfate (IS) to induce NOX4 mRNA and activity in endothelial cells,[41,58] leading to the increase of ROS and activation of MCP-1.[59] In a hypertensive rat model, IS induced oxidative stress with increased expression of NOX4, increased levels of 8-oxo-2'-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) in the heart.[60] One study reported IS-induced endothelial dysfunction in CKD patients, with *in vitro*

evidence for increased oxidative stress through, among others, increased NOX4 activity.[42] Advanced glycation endproducts (AGEs) and homocysteine, both known to accumulate in uremia, prompt ROS generation and activation of NOX.[61,62] Iron sucrose infusion induces endothelial cell dysfunction and LDL oxidation by Nox-activation in 5/6 nephrectomized mice and increases atherogenesis in ApoE $^{-}/^{-}$ uninephrectomized mice. In patients with CKD increased circulating mononuclear $^{\cdot}O_2^{-}$ production, expression of soluble adhesion molecules, and mononuclear–endothelial adhesion were found.[63]

II. Endothelial nitric oxide synthase (eNOS)

The nitric oxide synthases release the potent vasodilator NO by catalyzing the oxidation of L-arginine to L-citrulline. The normal function of eNOS requires dimerization of the enzyme and the presence of the essential cofactor (6R)-5,6,7,8-tetrahydro-l-biopterin (BH4), one of the most potent naturally occurring reducing agents. Under certain circumstances eNOS becomes 'uncoupled' with subsequent reduction of molecular oxygen instead of electron transfer to L-arginine, and hence generation of O_2. Superoxide anion and NO then combine rapidly to form $ONOO^{-}$, which in turn gives rise to a broad array of oxidants, leading to lipid and protein oxidation reactions.[22,24] Uncoupling of eNOS has important implications for oxidative stress in the vascular bed and can be caused by several mechanisms. It can result from reduced levels of BH4 or from the accumulation of asymmetric dimethylarginine (ADMA).[64,65] The latter molecule is a naturally occurring L-arginine analogue found in plasma and various types of tissues, acting as an endogenous NO synthase inhibitor *in vivo*. It is generated by the enzyme protein arginine N-methyltransferase (PRMT, type I) and degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). The activities of both PRMT and DDAH are redox sensitive: oxidative stress has been shown to increase PRMT(s) and decrease DDAH activity, thereby leading to an increase of ADMA concentrations.[66] In addition, plasma ADMA levels increase due to reduced renal excretion in CKD. ADMA has been shown to be associated with endothelial dysfunction and vascular disease in CKD.[43,67,68]

III. Xanthine oxidoreductases

Hyperuricemia and oxidative stress participate in the pathophysiology of hypertension and its complications. The Xanthine oxidoreductases exist both as a dehydrogenase (XDH) and oxidase (XO) of which the former is predominant. Upon the oxidation of hypoxanthine and xanthine to uric acid, the dehydrogenase generates NADPH whereas the oxidase gives rise to $^{\cdot}O_2^{-}$ and H_2O_2. The XDH can be converted into XO through the oxidation of critical thiol groups and subsequent proteolytic cleavage.[69] Oscillatory shear stress, a condition occurring at arterial sites prone for atherosclerosis, has been found to suppress XDH in favor of XO with a subsequent important increase in ROS

generation.[70] One study, by Gondouin et al., revealed an increased XO activity in hemodialysis patients. Moreover, XO activity was shown to be an independent predictor of cardiovascular events in both CKD and HD patients, regardless of uric acid levels. Uric acid was not associated with cardiovascular events.[71] The influence of decreased renal excretion of uric acid with subsequent hyperuricemia in CKD patients will be discussed below.

IV. Myeloperoxidases

Myeloperoxidase (MPO) is a heme-containing enzyme present in azurophilic granules of leukocytes. It catalyzes the reaction of H_2O_2 with halides to produce the secondary oxidant hypochlorous acid (HOCl). The latter can generate reactive oxidizing and chlorinating species that damage important biomolecules. Since MPO is the only human enzyme able to generate HOCl, chlorinated protein residues such as 3-chlorotyrosine are considered specific biomarkers of MPO activity. MPO most likely plays a role in atherosclerosis.[72] Clinical trials have demonstrated a correlation of circulating MPO levels and MPO-derived oxidized molecules with coronary artery disease (CAD) and clinical events.[27,28] MPO contributes to oxidative modification of LDL by catalyzing lipid peroxidation.[73] More recent studies show that MPO, by interacting with the nitric oxide (NO) pathway, can serve as a "NO oxidase". This aberrant function of MPO may provide another link for its positive association with CVD.[27] In hemodialysis populations plasma proteins contained 3-chlorotyrosine, the biomarker of MPO activity, whereas this was not found in age- and sex matched controls. Also, during hemodialysis sessions higher plasma MPO levels were measured.[74] This has been linked to leucocyte activation resulting from membrane incompatibility.[75] MPO levels in maintenance hemodialysis patients were shown to independently predict 3-year mortality risk after adjustment for multiple inflammatory markers and well-known clinical outcome-related variables.[76]

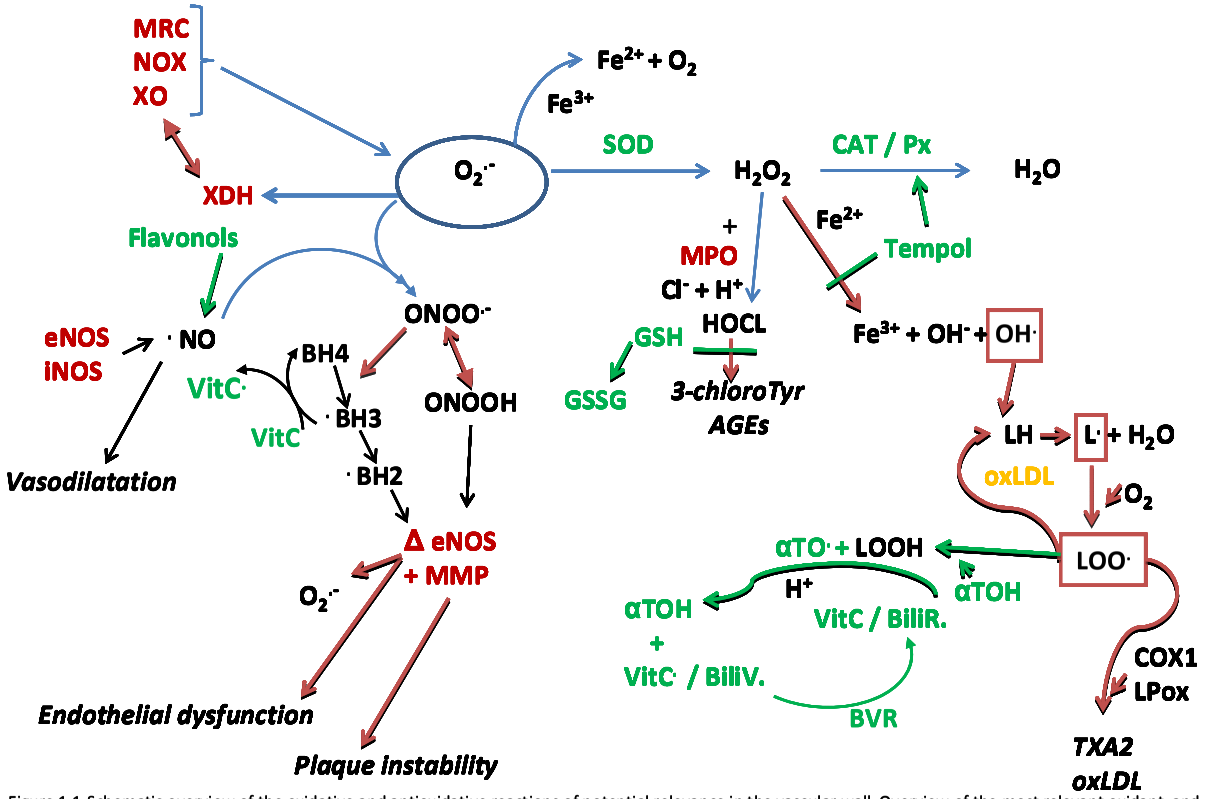

Figure 1.1 Schematic overview of the oxidative and antioxidative reactions of potential relevance in the vascular wall. Overview of the most relevant oxidant and antioxidant pathways in the vascular bed and their interactions. Green arrow :antioxidative reaction; Green line : inhibition of the reaction by antioxidative mechanism. Red arrow : Pro-oxidative reaction. Abbreviations: αTOH tocopherol, AGEs advanced glycation endproducts, BH hydro-l-biopterin, BVR Biliverdin Reductase, CAT catalase, COX cyclooxygenase, GSH/GSSG Glutathion, HOCl Hydrochlorou acid, Lpox lipoxygenase, MMP matrix metalloproteinase, MPO myeloperoxidase, MRC mitochondrial respiratory chain, NOS nitric oxide synthase, NOX NADPH oxidase, ONOO peroxynitrite, ONOOH peroxynitrous acid, VitC Vitamin C, XO Xanthine oxidase, XDH Xanthine dehydrogenase

V. Lipoxygenases

Lipoxygenases give rise to biologically active lipids, including prostaglandins, thromboxanes (TXA) and leukotrienes (LT), being metabolites of arachidonic acid and similar fatty acids. Examples of lipoxygenases are cyclooxygenase I (COX 1) and non-heme iron-containing lipoxygenases, such as 12- or 15-lipoxygenase. Lipoxygenases are activated by 'seeding peroxides' that oxidize the heme-iron to its active form 'Fe^{3+}'. The end-products of COX 1, such as prostacyclin and thromboxane A_2, mediate vasodilation and vasoconstriction/platelet aggregation, respectively. Furthermore, both cyclooxygenases and lipoxygenases contribute to LDL oxidation and nonenzymatic lipid peroxidation.[13] To our knowledge, there are no data on lipoxygenases and atherosclerosis in the context of CKD. Although not enzymatic end-products of lipoxygenases, 2 end-products of lipid peroxidation are worth mentioning: Malondialdehyde (MDA), is the principal and most studied product of polyunsaturated fatty acid peroxidation. This aldehyde is a highly toxic molecule and should be considered as more than just a marker of lipid peroxidation. Shao and colleagues have shown a role for MDA in the generation of dysfunctional HDL, by modifying and cross-linking specific residues in Apolipoprotein-A1, the main HDL protein[77,78]. MDA levels are elevated in CKD, and are associated with CVD in hemodialysis populations.[68,79] It remains however unclear if MDA contributes to the impaired antioxidant function of HDL in CKD/HD.[80,81] F2-isoprostanes are prostaglandin-like molecules generated by the free radical-induced peroxidation of arachidonic acid, independent of the cyclooxygenases. F2-isoprostanes have been determined to be both biomarkers and mediators of oxidative stress in numerous disease settings. F2-isoprostanes are reported to be increased in CKD, but no association with CVD has been shown yet.

VI. Mitochondrial respiration

The mitochondrial respiratory chain is responsible for the generation of ATP by oxidative phosphorylation of $NADH/FADH_2$, using O_2 as the terminal electron acceptor. Thereby, it also constitutes the major source of ROS in most mammalian cells. As a by-product of the electron flow through the respiratory chain complexes, 1-4% of molecular oxygen, determined to be reduced to water at complex IV, will be incompletely reduced to $O_2^{\cdot -}$.[82] Within mitochondria $^{\cdot}O_2^-$ production occurs mainly at complex I and III, NADH dehydrogenase and ubiquinone-cytochrome bc1 respectively. The steady state levels of the generated $^{\cdot}O_2^-$ depend on the activity of manganese superoxide dismutase (Mn-SOD or SOD2) which is responsible for the dismutation of O_2^- to H_2O_2. The resulting H_2O_2 is then further reduced to water by catalase. With respect to the vasculature, mitochondrial reactive oxygen species (mROS) have been shown to be highly involved in endothelial dysfunction, lipid oxidation, monocyte

infiltration and activation (reviewed in[83]). Also, complications of diabetes and obesity have been linked to the increased mitochondrial $^{\cdot}O_2^-$ generation. There is no direct evidence for increased mitochondrial ROS generation and atherosclerosis in the context of CKD. However, uremia as well as dialysis have been shown to lead to mitochondrial dysregulation.[34,84] Granata et al. found in a transcriptomic analysis of peripheral mononuclear cells that CKD/HD patients display a different gene expression profile of the oxidative phosphorylation system as compared to healthy subjects, suggesting an impaired mitochondrial respiratory system.[84]

VII. Other forms of oxidants

Free transition metals like copper and iron, in the presence of hydroperoxides, are strong catalysts for oxidation reactions. They can initiate lipid peroxidation by cleavage of LOOH to lipid alkoxyl radicals. Their exact role in atherosclerosis remains controversial. In general, the *in vivo* concentration of free metals was estimated to be too low to contribute to the atherosclerotic process, based on studies in hemochromatosis patients.[85] However, Sullivan et al. found the atheroprotective aspect of hemochromatosis to be linked to decreased hepcidin levels and hepcidin itself to be atherogenic and plaque destabilizing.[86,87] In CKD patients, and certainly in hemodialysis patients, intravenous (iv) iron supplementation is a commonly required therapy for successful anemia management.[88] Iv iron has been identified as a possible culprit of oxidative stress. Khuo et al. found iron sucrose to induce endothelial dysfunction and ROS generation in mice with renal dysfunction as well as in circulating mononuclear cells from CKD patients.[89] Experimental as well as epidemiological data remain controversial, however.[90] Randomized clinical trials studying cardiovascular events following different schedules of iv iron therapy in CKD/HD patients would be needed to better define its role in clinical outcomes. Copper is transported by albumin to the liver where it is incorporated in ceruloplasmin for transport to various tissues. Ceruloplasmin has ferroxidase capacity required for iron incorporation into ferritin.[91] It has been reported to induce and facilitate LDL oxidation by free metals.[92,93] Recently, Kennedy et al. reported increased ceruloplasmin levels in CKD patients, associated with increased risk of long-term adverse cardiovascular events, even after multivariable model adjustment for traditional clinical and biologic risk factors.[94]

B. Antioxidant defense mechanisms in the vessel wall

To understand the contribution of oxidative stress to atherosclerosis, it is also important to consider the available antioxidative mechanisms in the vascular bed. Many antioxidants may indeed prevent or delay oxidation of substrates.

I. Nonenzymatic antioxidants

The extracellular space, including the plasma, contains only little enzymatic antioxidants and therefore relies mainly on nonenzymatic antioxidants. Halliwell and others have shown that protein-associated thiols and ascorbic acid form the major antioxidant defense in plasma.[95] CKD has been shown a condition with a generalized depletion of dietary antioxidants such as vitamin C and vitamin E, which is partially due to dietary restrictions of, among others,fresh fruits and vegetables.[96] Zinc and selenium are both trace elements with key functions in antioxidant defense, as they act as cofactors of enzymatic systems such as glutathione peroxidase. Both zinc and selenium have been reported to have decreased levels in dialysis patients.[97]

a. Proteins

Albumin is well-known for binding a large variety of molecules, including fatty acids, drugs, hormones, and metal ions. It has several antioxidative capacities. First, albumin binds transition metal ions (copper and iron essentially). Indeed, albumin possesses several metal binding sites and inhibits *in vitro* lipid peroxidation reactions at concentrations similar to those in the vascular bed.[93] Second, it is a major protein target for carbonyl formation and acts as a binding protein for oxidation end-products such as AGEs.[98,99] Finally, albumin is the major source of extracellular **thiols**, which are the most important antioxidant mechanisms of the extracellular space. These thiol groups act as ROS scavengers and constitute the primary defense against secondary oxidants such as HOCl.[100] Himmelfarb and others have shown that plasma protein thiols are extensively oxidized in CKD patients, suggesting antioxidative depletion.[101] Additionally, in CKD patients, hypoalbuminemia is a frequent feature as a consequence of inflammation and malnutrition. Taking this all together, the pathophysiology behind the clinical findings in cross-sectional studies showing a strong correlation between hypoalbuminemia and cardiovascular mortality in CKD patients is conceivable.[98, 102]

b. Non-proteinaceous antioxidants

Next to the antioxidative proteins, several low molecular weight compounds contribute to the antioxidative defense mechanisms of the human body. Glutathione (GSH) is an ubiquitous non-proteinaceous thiol involved as a cofactor for GPx, in ascorbate metabolism and in protein folding. Moreover it functions as a free radical scavenger. Given its pluripotency, GSH will be discussed in more detail in the sections on the specific antioxidant systems it is involved in. Other than GSH, several dietary antioxidants as well as compounds synthesized in the body, either water- or lipid soluble, have been described.

i. Water soluble antioxidants

Dietary antioxidants include vitamin C or ascorbic acid, flavonoids and polyphenols. The latter two were believed to hardly contribute given their poor absorption and high metabolism.[13] However, during the last decade, the antioxidant potential of polyphenols is gaining more and more attention since promising results have been reported.[103-105] Uric acid and bilirubin are endogenous compounds, also contributing to the antioxidative arsenal. Finally, tempol is a new promising antioxidative nitroxide.

- Ascorbate (vitamin C)

Vitamin C is ubiquitous in biological fluids and present as ascorbate at physiologic pH. It functions as a cofactor for several enzymes participating in hydroxylation reactions. Considering processes in the vascular bed, ascorbate is important for the synthesis of collagen. In case of ascorbate deficiency, collagen formation is hampered resulting in, amongst others, fragile blood vessel formation. Despite its *in vitro* pro-oxidative characteristics (through the redox cycling of iron and generation of hydroxyl radicals by the Fenton reaction), ascorbate generally protects molecules against oxidative damage *in vivo* in human plasma.[106-108] Many antioxidative, anti-atherosclerotic activities of vitamin C have been described[109]: Generally, ascorbate is regarded a radical scavenger that can be easily recycled by glutathione-dependent reducing enzymes. It has been shown to inhibit LDL peroxidation, both by scavenging primary oxidants, but also through the protection against secondary oxidants, albeit not completely, such as HOCl and $ONOO^-$ [13]. Next to its scavenging role, it acts as a synergistic antioxidant: ascorbate contributes to the reduction of α-$TO^·$ to α-TOH and is involved in the maintenance of cellular glutathione through the reduction of the glutathione thiyl radical.[110] There is clinical evidence for the atheroprotective effect of vitamin C as well. In patients with unstable coronary disease lower plasma concentrations of ascorbate have been found.[111] Vitamin C supplementation is associated with significant improvement of endothelial function in subjects with cardio-metabolic disorders, as recently shown in a meta-analysis by Ashor et al.[112] In unsupplemented CKD and dialysis patients, ascorbate plasma levels are low, despite dietary intake close to the recommended daily amounts.[113] Increased water diuresis, osmotic diuresis and loop diuretics all increase urinary ascorbate losses in CKD patients. About 200 mg ascorbate per week is lost through dialysis.[37,114] A study by Tarng et al. in 60 hemodialysis patients showed reduced lymphocyte 8-OHdG levels and intracellular ROS production following IV supplementation of 300mg vitamin C postdialysis, three times per week.

- Flavonoids and Polyphenols

Dietary polyphenols present in wine, green tea, cocoa and fruits are mild antioxidants. In the late 90's their antioxidant effects were considered as too small to be clinically relevant.[13] In recent years however, they gained more interest and it is believed now that plant-derived polyphenols may offer long-term benefits by, among others,lowering ROS.[104] Cocoa flavanols (CFs), like epicatechin, catechin and the procyanides have been substantiated by the European Food Safety Authority as biologically active food constituents.[115] The exact mechanisms by which they exert beneficial effects are incompletely understood. It is believed to be at least partially through an increase of NO bio-availability and increased nitrosothiol plasma levels next to influences on cellular signal cascades, gene expression, and enzyme activities.[104] Interventional studies have demonstrated beneficial effects from CFs on blood pressure regulation and endothelial function.[116,117] Rassaf et al., in a randomized double–blind placebo-controlled trial in patients with ESRD on hemodialysis, found sustained attenuation of endothelial dysfunction after the ingestion of CFs (900mg/day). Moreover, CFs mitigated HD–induced vascular dysfunction and decreased diastolic blood pressure, suggesting amelioration of microvascular function.[118]

- Uric Acid

Uric acid is generated by oxidizing hypoxanthine and xanthine catalyzed by Xanthine Dehydrogenase or Oxidase (cfr earlier (section 5A.III). At physiological pH uric acid circulates as urate mono-anion. Urate can function as a scavenger for both primary as well as secondary radicals. It is believed that one of the biological functions of uric acid may be that of an antioxidant.[119] For instance, urate binds transition metals and may protect in this way against LDL lipid peroxidation in the vascular wall. On the other hand, elevated uric acid levels are very often seen in patients with metabolic syndrome and thus found to be correlated with CVD. Additionally, in CKD patients, reduced renal urate excretion leads also to hyperuricemia and this has been related to renal function impairment.[120] A detailed discussion of the conflicting data regarding hyperuricemia, CKD and CKD-related CVD is beyond the scope of this overview (Reviewed in[120-122]).

- Bilirubin

Bilirubin is generated by the degradation of heme and is a strong reducing agent. In view of its lipophilic nature, it is predominantly bound to albumin in the extracellular fluids. Both free and albumin-bound bilirubin are able to reduce α-TO$^{\cdot}$ to α-TOH and to inhibit LDL lipid peroxidation.[123] Bilirubin is also a good scavenger for ROS such as peroxyl radicals. The latter oxidize bilirubin to biliverdin, which is then reduced by biliverdin reductase to bilirubin, creating a recycling system with a large antioxidant

scavenging-potency.[124] A number of data are available on the antioxidant effects of bilirubin in the context of CVD. Bilirubin improves myocardial function in a model of ischemia/reperfusion[125] and is able to modulate inflammatory status in a LPS treated rat model, mitigating endothelial activation[126]. Hyperbilirubinemic Gunn Rats are protected against Angiotensin II and balloon-induced vascular injury.[127,128] In addition, a substantial amount of clinical studies demonstrated serum bilirubin to be a protective marker in atherosclerosis and coronary artery disease.[129-132] Gilbert's syndrome (GS) for example, a condition of mild and benign hyperbilirubinemia, has recently been shown to be clearly associated with protection from CVD.[133,134] In the CKD population, no experimental studies regarding the effect of bilirubin exist. Nevertheless, in a clinical observation Tbahriti et al. found decreasing bilirubin levels in parallel with lowering of renal function.[96]

- Tempol

Cyclic nitroxides are worth mentioning as well. They constitute a group of diverse stable free radicals that have unique antioxidant properties. Tempol is a water-soluble and cell permeable superoxide dismutase mimetic which scavenges free radicals such as superoxide anions and hydroxyl radicals. Like endogenous SOD, it acts as a catalyst and is not consumed in the process of dismutation of $^{\cdot}O_2^-$ to H_2O_2 and oxygen. Additionally, the nitroxides were shown not only to act as SOD mimics, but also to confer catalase-like behavior, inhibit lipid peroxidation and prevent the generation of $^{\cdot}OH^-$ radicals by the inhibition of Fenton reactions.[135] Nitroxides, when administered to hypertensive animals, result in a decrease in mean arterial blood pressure.[136] This effect was demonstrated to occur via a NO-related mechanism in *in vitro* experiments performed on cultured endothelial cells and in animal studies.[137] Recently, tempol has also been shown to protect against atherosclerosis in Apo $E^-/^-$ mice models of metabolic syndrome and dyslipidemia. In fructose fed mice, tempol reduced plaque inflammation and plaque size and this was associated with a reduction in oxidative stress and p47phox expression, suggesting the inhibition of the NOXes.[138] In the high-fat fed mice, tempol supplementation increased plaque collagen content, decreased lipid content and increased macrophage numbers, suggestive for a plaque stabilizing effect.[139] In the context of CKD-related atherosclerosis, tempol has not been extensively studied. Yamada et al. however, showed tempol to reduce arterial medial calcification in a rat-model of CKD-MBD.[140] In addition, there is a substantial amount of data showing the preservation of kidney function and salt balance in animal models of hypertension and kidney failure. The most pronounced effect of tempol is seen in Dahl salt-sensitive rats where the drug not only prevents blood pressure increase through the regulation of NAD(P)H oxidases, but also the development of glomerulosclerosis, proteinuria and the

associated loss of renal function.[141-143] Ding et al. confirmed these findings in a 5/6th nephrectomized mouse model showing tempol supplementation to attenuate oxidative stress, inflammation, fibrosis and deterioration of the remnant kidney function.[144] Taking these findings together, tempol can be considered a promising agent for future studies on the prevention of both progression of renal failure and the associated CVD.

ii. Lipid Soluble antioxidants

In addition to aqueous antioxidants, lipid soluble antioxidants play an important role in biological tissues. Tocopherols and Ubiquinones, residing mainly in lipoproteins and cell membranes, are the most important representors of this group.

- Vitamin E

Vitamin E is a nutritional term referring mostly to α-tocopherol, the most active of the eight different, naturally occurring tocopherols with variable vitamin E-activity. Being fat-soluble, α-TOH localizes mainly in cell membranes and lipoproteins. Consequently, it is the major antioxidant, central to the prevention of radical-induced LDL lipid peroxidation, in which it reacts rapidly with formed peroxyl radicals ($LOO^{\cdot}$) to generate α-$TO^{\cdot}$. The resulting α-$TO^{\cdot}$ is relatively nonreactive. In addition to scavenging peroxyl radicals, α-TOH can also react with singlet oxygen and some secondary radicals such as HOCL and $ONOO^{-}$. Ascorbate rapidly reduces α-$TO^{\cdot}$ back to α-TOH. Vitamin E supplementation was shown to reduce oxidative stress, inflammation and athero-sclerosis in patients with coronary artery disease.[145,146] In the CKD population, one group demonstrated lower levels of vitamin E[96] as compared to the general population, but generally normal serum levels of α-Tocopherol are reported[31,147,148] despite impaired erythrocyte and mononuclear cell content.[149] Numerous interventional studies with vitamin E supplementation have been conducted. Beneficial effects of oral supplementation such as a reduction in oxidative stress and improvement of LDL resistance to oxidation were reported.[149,150] Moreover, a reduction in composite cardiovascular events and myocardial infarction has been described in the SPACE study in HD patients.[151] The difference between alpha- and gamma-Tocopherol in terms of biological activity remains incompletely understood: both isomers showed protective properties in the general as well as CKD population.[152-155]

- Coenzyme Q_{10}

Coenzyme Q_{10} or Ubidecaquinone belongs to the ubiquinones family: fat soluble molecules containing a benzoquinone structure with 1 to 12 isoprene units. Coenzyme Q_{10} has 10 isoprene units, is the predominant ubiquinone in humans and is found in

almost every cell membrane and lipoproteins. It exists in three oxidation states: the fully reduced ubiquinol ($CoQ_{10}H_2$), the ubisemiquinone radical intermediate ($CoQ_{10}H^{\cdot}$) and the fully oxidized ubiquinone (CoQ_{10}) form. It is incorporated in the electron transport chain where it plays an important role in mitochondrial respiration and ATP production.[82] Like α-TOH, Coenzyme Q_{10} plays a key role in the prevention of lipid peroxidation. Coenzyme Q_{10} supplementation is associated with significant improvement in endothelial function.[156] Only limited data are available in the context of CKD, and no evidence for decreased Coenzyme Q_{10} levels nor for a beneficial effect of Coenzyme Q_{10} supplementation exists.[157,158]

II. Enzymatic antioxidants

a. The 'classic antioxidative enzymes'

The classic antioxidative enzymes are mainly cell-associated proteins. Their sole function is to maintain the reducing tone within cells and keep the redox balance stable. They also regulate the maintenance of the extracellular (enzymatic and nonenzymatic) antioxidants such as glutathione. Most of these antioxidant enzyme systems have been studied in the context of CKD and many of them were found to be disturbed.[96] Interventional studies however are lacking to date.

- Superoxide dismutase (SOD)

The super oxide dismutases catalyze the conversion of $^{\cdot}O_2^-$ to H_2O_2 and O_2, after which H_2O_2 can be turned into water by catalase or the gluthatione peroxidase system. There are 3 isoforms. First, copper-zinc SOD (SOD1) resides in virtually all cells where it is located in the cytosol with some activity in lysosomes, nucleus and between the inner and outer mitochondrial membrane. Second, manganese-dependent SOD (SOD2), which is located mainly in the mitochondria. And finally, extracellular SOD (SOD3) which is another copper-zinc SOD that is mainly bound to heparan sulfate proteoglycans in the glycocalyx and connective tissue matrix of the vascular wall.[159] The copper-zinc enzymes function through alternating oxidation and reduction of the copper ion and are pH independent, in contrast to the manganese-dependent enzyme which is less active at alkaline pH. The synthesis and activity of SOD3 is modulated by cytokines, growth factors and oxidants. A key function of SOD3 is thought to protect against $^{\cdot}O_2^-$ induced inactivation of endothelial cell-derived NO. Of note, the reaction of $^{\cdot}O_2^-$ with $NO^{\cdot}$ is about three times faster (6.7×109 mol L^{-1} s^{-1}) than its reaction with SOD.[160] Thus high SOD expression and activity are required for effective protection. There is relatively little data from experimental and clinical studies regarding SOD and atherosclerosis. Normal SOD levels and activity were reported in patients with coronary

disease.[161,162] Despite this, higher SOD2 activity related to a functional SOD2 polymorphism, has been shown to protect against coronary artery disease and myocardial infarction.[163] Another argument in favor of the protective effect of increased SOD activity can be derived from the beneficial effects of tempol, as described earlier (section 4B.I.b.i). In CKD, impaired SOD activity has been repeatedly reported by several groups.[96,164-166] This could at least partially be explained by a concomitant Zinc (Zn) deficiency with subsequent SOD impairment.[165] Indeed, Zn-deficiency is well described in hemodialysis patients.[97] Moreover, next to its role as a cofactor of SOD, Zn is also required for the upregulation of the zinc-finger protein A20, which inhibits inflammatory pathways through the inhibition of TNFα and IL1β.[167] Shen et al. observed zinc deficiency to increase oxidative stress and NF-κB DNA-binding activity, induce cyclooxygenase-2 (COX-2), and E-selectin gene expression, as well as monocyte adhesion in cultured endothelial cells, suggesting a key role in inflammatory diseases such as atherosclerosis.[168] In agreement with these findings, Lobo et al. demonstrated in a small study of hemodialysis patients an inverse correlation between Zn-levels and lipid peroxidation and inflammation as indirect markers of CVD.[169,170]

- Catalase and peroxidases

Two enzymes metabolize H_2O_2 resulting from SOD or generated by, among others, xanthine oxidase. Catalase (CAT) directly decomposes H_2O_2 to water and O_2, whereas the peroxidases (PX) use H_2O_2 to oxidize another substrate, such as glutathione (GSH). Mostly, the GSH-PXs cooperate with CAT for the decomposition of H_2O_2 to H_2O and oxidized glutathione (GSSG), which is then reduced by glutathione reductase. GSH-PXs require selenium (Se) for their activity, which actively participates in the catalytic reaction. Four subtypes of Se-containing peroxidases exist, each contributing to the antioxidant defense to H_2O_2 and hydroperoxides.[13]

A role for GSH-PX in atherosclerosis has been demonstrated. In patients with suspected coronary artery disease, low levels of activity of red-cell GSH-PX were independently associated with an increased risk of cardiovascular events.[171]

Several groups also reported disturbed GSH-PX function in CKD.[166,172,173] Plasma GSH-PX is decreased in CKD and dialysis and GSH/GSSG ratio is disturbed early in the course of CKD. Ceballos-Picot et al. reported gradually increasing alterations of the glutathione dependent antioxidant system along with the degree of renal failure, further deteriorating in patients on peritoneal dialysis and culminating in hemodialysis patients in whom an almost complete abolishment of GSH-PX activity was observed.[166] This finding was accompanied by low levels of selenium (Se). Indeed, Se functions as an antioxidant, primarily in the form of the selenoproteins. At least 30 selenoproteins have been identified, including GSH-PX, selenoprotein P, thioredoxin reductase and

selenophosphate synthetase.[174] Se-deficiency is a well-known phenomenon in CKD and dialysis patients and has been associated with increased oxidative stress and mortality, albeit mainly from infectious diseases.[172,175] Girelli et al. also found an association between Se-levels and CVD. The mechanism behind Se-deficiency is not completely unraveled. Loss of Se through dialysis was suggested but is rather unlikely since Se is bound to high-mass molecules and thus not easily dialysed. Furthermore, Se-deficiency was also reported in predialysis CKD patient populations. A decreased intake or decreased intestinal absorption are two remaining plausible explanations.[173,176] Se-supplementation has been recently studied by several groups.[177,178] Salehi and colleagues found a decrease in the surrogate markers of malnutrition, inflammation and oxidative stress and hypothesized that this could contribute to protection against CVD and mortality in HD patients when using high dose-supplements.[179] Clearly, this hypothesis needs to be further evaluated in larger studies with longer durations.

When it comes to catalase, there are only few and conflicting data: Whereas Mekki et al. report a decreased CAT activity, worse in HD/PD patients as compared to predialysis CKD patients,[180] the group of Shurtz-Swirski et al. found augmented levels in dialysis patients, together with the decreased SOD activity.[165]

- Thiol-disulfide oxidoreductases

The ratio of GSH to GSSG regulates the activity of protein folding enzymes, the so-called 'protein disulfide isomerases', found on the cell surface and proposed to be involved in the maintenance of the redox balance by the regulation of protein thiols, reactive disulfide bonds and transnitrosation reactions.[181] These enzymes belong to the thiol-disulfide oxidoreductases and include thioredoxin and glutaredoxin. Thioredoxin acts in conjunction with thioredoxin reductase and is a 12kD protein, secreted by most cell types, with a redox-active dithiol/disulfide in the active site. It is secreted into the circulation in response to oxidative stress.[182] The oxidoreductases contribute to the antioxidant defense mechanisms in many different ways and may play a central role in cellular redox signaling.[183] Little is known on the oxidoreductases and thiorexodin in the context of CKD. One group reported increased levels in subjects with mildly reduced renal function as compared to controls.[184] Another study, by Kasuno et al., demonstrated a protective role of thioredoxin overexpression in ischemia/reperfusion mediated injury in a mouse model.[185]

b. Antioxidative response element-driven enzymes – Heme oxygenase-1

Phase 2 genes, encoding for enzymes like heme oxygenase-1 (HO-1) and glutathione reductase, are regulated by upstream antioxidant response elements (ARE), which are activated by the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2).[186] Regulation of Nrf2, a leucine zipper transcription factor, occurs through binding to Keap1 and the repression factor Bach1. Under Basal conditions, Keap1, a cysteine rich protein associated with the actin cytoskeleton, binds firmly to Nrf2, anchoring the transcription factor in the cytoplasm and targeting it for ubiquitination and proteasomal degradation, hereby repressing Nrf2 activity. Activation of the phase 2 genes occurs through the disruption of the Keap1-Nrf2 complex. Inducing agents modify specific thiol groups of Keap1, causing a conformational change which renders Keap1 unable to bind Nrf2. This allows Nrf2 to migrate to the cell nucleus and bind to the ARE sites on the promotor region where it accelerates the transcription of the phase 2 genes. ROS and other oxidizing agents belong to the inducers of the phase 2 genes and both HO-1 as well as glutathione reductase can thus be 'switched on' in times of increased oxidative stress.

Genes affected by Nrf2 provide not only direct antioxidants, they also encode enzymes that directly inactivate oxidants, increase levels of glutathione synthesis and regeneration and stimulate NADPH synthesis.[187,188] Amongst these genes, heme oxygenase-1 has been intensively studied in the context of atherosclerosis.[189-191]

There are 2 functional heme oxygenases in the human body: an inducible (HO-1) and a constitutively active isoform (HO-2). HO-3, a pseudogene, was found only in rats. HO-1 is considered an early stress-responsive protein, and thus far, not expressed constitutively in normal tissues, apart from the spleen, Kupffer cells in the liver, cerebellary Purkinje cells and regulatory T cells. However, it can be strongly upregulated in virtually all cell types.[192] Being an early stress-responsive protein, the HO-1 isoform can be induced by a variety of agents that cause oxidative stress including heme, lipopolysaccharide (LPS), H_2O_2, heavy metals, ultra-violet radiations, cytokines and endotoxins.[192] But also nitric oxide and carbon monoxide (CO) can induce HO-1.[193]

Determining the rate-limiting step in the degradation of heme, HO-1 catalyzes the oxidative detoxification of excess heme resulting in equimolar amounts of free iron (Fe^{2+}), CO and biliverdin, the latter being rapidly reduced to bilirubin by the NADPH-dependent biliverdin reductase. All products formed in this process are potentially beneficial, since they mediate anti-inflammatory, antioxidant and anti-apoptotic effects. The overall effect can be summarized as the removal of the pro-oxidant

molecules heme and iron with, at the same time, generation of the antioxidants biliverdin and bilirubin and the release of CO.

The end-products of HO-1 activity exert protective effects, of which many result in protection against atherosclerosis (Figure 1.2). The possible beneficial effects of HO-1 through the generated end-products biliverdin/bilirubin, Iron and carbon monoxide are visualized in Figure 1.3.

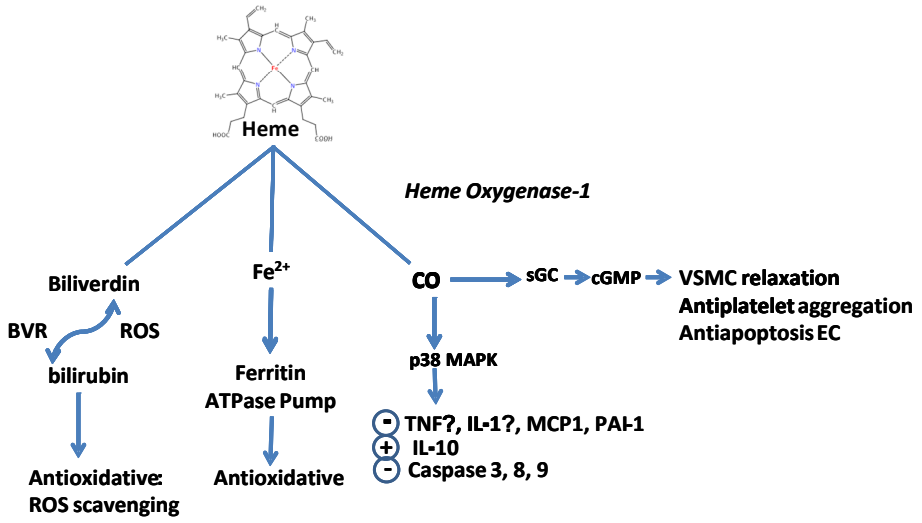

Figure 1.2 Role of heme oxygenase-1 in atherosclerosis. Schematic overview of the most relevant effects of heme oxygenase-1 in the vascular wall. Abbreviations are: BVR biliverdin Reductase, CO Carbon monoxide, EC endothelial cells, Fe2+ iron, IL interleukin, MCP1 monocyte chemoattractant protein 1, PAI-1 plasminogen activator inhibitor type 1, ROS Reactive oxygen species, sGC soluble Guanylyl cyclase, TNFα Tumor necrosis factor α,VSMC vascular smooth muscle cells,

The protective effects of **bilirubin** were described earlier (section 4B.I.b.i).

Carbon monoxide has a pluripotent role in the vascular bed. First, early studies on CO pointed NO-like activities, such as vasomotor tone modulation. Indeed, relaxation of smooth muscle cells and reduction of endothelin-1 and Platelet derived growth factor (PDGF) in the endothelial cells of the arterial wall have been described.[194-196] Second, CO has been shown to influence many signaling pathways resulting in the induction of anti-inflammatory processes (IL-10) and inhibition of inflammation (TNFα, IL1-β).[197] At the same time, it suppresses thrombosis and platelet aggregation, partially through the inhibition of oxLDL mediated plasminogen activator inhibitor type1 (PAI-1) expression. Last, but not least, CO has well described anti-apoptotic effects in different cell-types, through various signaling pathways.[192]

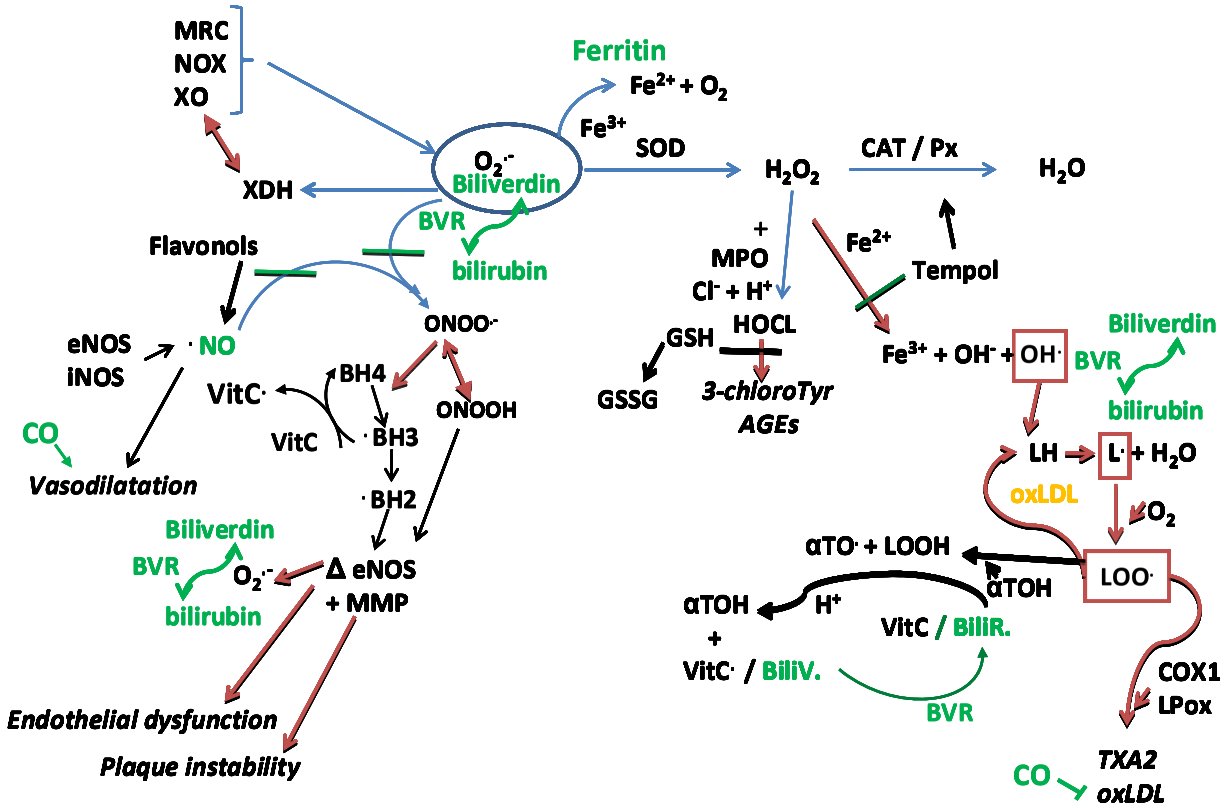

Figure 1.3 Schematic overview of the oxidative and antioxidative reactions of potential relevance in the vascular wall. Potential protective mechanisms of HO-1 on the oxidative/antioxidative reaction balance in the vascular wall. Effects of HO-1 induction and effects of the HO-1 mediated endproducts of degradation of heme are shown in green.

The released **free iron** by HO-1 is considered to rapidly induce the formation of ferritin and an ATPase pump that actively removes intracellular iron from the cell. The resulting modulation of the intracellular iron store and increased iron efflux are believed to be one of the mechanisms by which HO-1 confers resistance to oxidative stress.[198] Indeed, in the human case of HO-1 deficiency abnormalities in the reuse of iron and tissue iron deposition have been observed[199,200] and several studies have shown the cytoprotective effect of ferritin-induction by the release of iron from heme.[201]

The involvement of HO-1 in atherosclerosis was first demonstrated by Wang et al. Indeed, immunostaining of human atherosclerotic plaques showed the presence of HO-1 in macrophages, foam cells, endothelial cells and vascular smooth muscle cells whereas no HO-1 was shown in nonatherosclerotic arterial sections.[202] In HO-1$^{-}/^{-}$ ApoE$^{-}/^{-}$ mice accelerated and more advanced atherosclerotic lesion formation was seen as compared to HO-1$^{+}/^{+}$ ApoE$^{-}/^{-}$ mice, illustrating the anti-atherogenic properties of the enzyme.[203] Moreover, HO-1 is an important modulator of atherosclerosis, determining lesion progression and/or stabilization.[204]

The activity of HO-1 is influenced by genetic factors. In the promotor region of the HO-1 gene, which is located on chromosome 22q12 and consists of four introns and five exons,[205] functional polymorphisms have been found[206]: a T(–413)A single nucleotide polymorphism (SNP rs2071746)[207,208] and a (GT)n dinucleotide repeat polymorphism. Data on the T(-413)A SNP are scarce and conflicting.[207-209] The (GT)n polymorphism, however, has been studied extensively. It consists of variable lengths of a purine–pyrimidine alternating sequence. Longer GT repeat areas acquire the potential to assume Z-DNA conformation, a lefthanded double-helix structure, thermodynamically unfavorable compared to the B-DNA conformation. This Z-DNA conformation has been described as negatively affecting transcriptional activity.[210] Longer (GT)n repeats have been proposed, therefore, to result in lower HO-1 expression and activity, and this was confirmed by luciferase promoter constructs and transient transfection assays in different cell lines.[211,212] In comparison, constructs with lengths of <27 repeats showed increased HO-1 basal promotor activity in response to oxidative triggers.

HO-1 has been intensively studied in renal ischemia-reperfusion in the context of renal transplantation,[213-216] in Acute Kidney Injury (AKI) models[217] and also in relation to arteriovenous fistula maturation.[218-220] So far, however, it is unknown how chronic uremia affects the expression and activity of HO-1, in particular with regard to atherosclerosis and inflammation.

One might expect HO-1 transcription and activity to be stimulated in CKD as a consequence of the overall pro-oxidant state described earlier in this chapter. Alternatively, however, the uremic milieu – as it is the case for other enzymes (e.g.

dimethylarginine demethylaminohydrolase (DDAH)) – may interfere with normal HO-1 function and abrogate its antioxidant potential.
Limited evidence in a kidney injury model is in favor of the latter hypothesis. Kim et al. reported impairment of the Nrf2-Keap1 pathway and its target genes, amongst others HO-1, after six and twelve weeks of CKD in a rat remnant kidney model, despite clear evidence of increased oxidative stress and inflammation.[221] In addition, Preinduction of HO-1 using hemin, however, significantly alleviated tubulointerstitial fibrosis in a model of TGF-β mediated renal inflammation and fibrosis[222].

Summary

The CKD-related accelerated atherosclerosis has been attributed to the disturbance of the oxidant/antioxidant balance in CKD, favoring oxidative stress and subsequent oxidative damage.
Many pathophysiological mechanisms have been explored. Diverse mechanisms in CKD, both endogenous and exogenous, lead to increased activity of oxidative enzymes such as NOX, MPO and XO, the dysregulation of crucial enzymes such as the phenomenon of eNOS uncoupling or the accumulation of secondary radicals and transition metals. In addition many crucial antioxidative mechanisms have been found to be impaired. A promising field of renewed interest comprises interventions for simple dietary deficiencies and newly discovered compounds such as trace elements, flavonols and tempol. Still, many oxidant and certainly antioxidative systems remain relatively unexplored in the context of CKD. Amongst the latter are the ARE-driven enzymes, which can be 'switched on' in times of increased oxidative stress. These enzymes not only provide direct antioxidants, but also inactivate oxidants, increase levels of glutathione synthesis and regeneration and stimulate NADPH. A notable example of the ARE-driven enzymes is HO-1. This enzyme's overall antioxidative effects are supported by numerous findings on its atheroprotective potential in *in vitro* and *in vivo* animal investigations, as well as in human observational studies.
Despite all this, data on HO-1 in the particular context of CKD and CKD-related atherosclerosis is limited to non-existing and is an intriguing area of investigation.

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CHAPTER 2

Study objectives

Study objectives

The central purpose of the present project is to explore the particularly devastating atherosclerotic process in patients with CKD and the link with HO-1 expression and function in this process as well as in the chronic pro-inflammatory state of CKD patients.
So far, it is unknown how uremia affects the expression and activity of HO-1 in atherosclerosis and inflammation. One might expect HO-1 transcription and activity to be stimulated in chronic kidney disease (CKD) as a consequence of the overall pro-oxidant state. Alternatively, however, the uremic milieu – as it is the case for other enzymes (e.g. dimethylarginine demethylaminohydrolase (DDAH)) – may interfere with normal HO-1 function and abrogate its anti-oxidant potential.

Findings in kidney injury models are in favor of the latter hypothesis. Kim et al. reported impairment of the Nrf2-Keap1 pathway and its target genes, amongst others HO-1, after six and twelve weeks of CKD in a rat remnant kidney model, despite clear evidence of increased oxidative stress and inflammation.[7]

We hypothesize that HO-1 is involved in the accelerated atherosclerosis of CKD and that its activity may differ from what is seen in the general population. We further hypothesize that targeted modification of HO-1 might be a potential therapeutic pathway to mitigate the high cardiovascular morbidity and mortality of CKD.
the project has the following objectives:

I. To investigate morphological characteristics, expression of inflammatory markers and HO-1 of atherosclerotic lesions of patients with and without CKD, and search for correlations between clinical phenotype, degree of atherosclerosis, inflammation and cellular specificity.

II. To investigate how uremia affects the oxidative stress-induced endothelial cell toxicity and expression of the cytoprotective enzyme HO-1. To analyze how HO-1 induction contributes to endothelial cell protection from apoptosis.

III. To study the relation between HO-1 functional promoter polymorphisms and clinical outcomes in patients with and without CKD.

CHAPTER 3

Atherosclerotic lesion characterization in Chronic Kidney Disease : The 'vulnerable plaque' phenotype

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Submitted

Abstract

Chronic kidney disease (CKD) is characterized by accelerated atherosclerosis. At present, this is considered the consequence of both traditional and non-traditional cardiovascular risk factors such as increased oxidative stress. Heme oxygenase-1 (HO-1) is a known anti-oxidative and anti-atherogenic enzyme. We studied atherosclerotic plaque morphology, atherosclerotic markers and intraplaque protein and RNA expression of HO-1 in peripheral artery biopsies. Therefore, we included 153 patients planned for vascular surgery, stratified into either nonCKD or CKDG3-5D (CKD). Demographics, standard biochemical data, inflammatory markers (high sensitive CRP and interleukin-6), uremic toxins (indoxyl sulfate and p-cresyl sulfate) and markers of oxidative stress (F2-isoprostanes) were determined. In 86 of the 153 patients arterial lesion biopsies were available for histological phenotyping and 66 biopsies showed lesions of interest. Lesions of CKD patients expressed higher foam cell infiltration, increased monocyte chemoattractant protein-1 and activated caspase-3 expression and more neovascularization. There were more plaque complications. HO-1 mRNA was less frequently upregulated. In multivariate analyses, the estimated glomerular filtration rate was an independent predictor of neovascularization and plaque complications, independent of traditional cardiovascular risk factors. This study shows that atherosclerotic lesions in CKD patients express a vulnerable plaque phenotype and have a tendency to lower expression of the anti-atherogenic enzyme HO-1. Further investigation of the potential effects of CKD on the expression and function of HO-1 and other anti-atherogenic enzymes is warranted.

Introduction

Chronic kidney disease (CKD) is characterized by an accelerated atherosclerosis process as compared to the general population.[1] Recent data indicate that the impact of renal insufficiency on cardiovascular disease (CVD) already begins with minor renal dysfunction.[2] The risk of cardiovascular events increases as the estimated glomerular filtration rate (eGFR) declines.[3] Although CKD is often associated with cardiovascular risk factors such as diabetes, arterial hypertension (AHT) and dyslipidemia, the high occurrence of atherosclerotic vascular disease in CKD is not fully explained by these factors alone.[4] Several mechanisms have been postulated as contributing factors to the accelerated atherosclerosis of CKD, for instance increased vascular calcification, anemia, endothelial dysfunction and oxidative stess.[5] One enzyme in the human anti-oxidative arsenal, heme oxygenase-1 (HO-1), is also well studied in the context of atherosclerosis.

HO-1, the inducible enzyme and rate-limiting step in the degradation of heme, catalyzes the oxidative detoxification of excess heme resulting in equimolar amounts of free iron (Fe2+), biliverdin and carbon monoxide (CO). All products formed in this process are potentially beneficial, since they mediate anti-inflammatory, anti-oxidant and anti-apoptotic effects. HO-1 is involved in atherosclerosis as immunostaining shows its presence in macrophages, foam cells, endothelial cells and vascular smooth muscle cells of atherosclerotic arterial sections.[6] In HO-1$^{-/-}$ apoE$^{-/-}$ mice accelerated atherosclerotic lesion formation was seen as compared to HO-1$^{+/+}$ apoE$^{-/-}$ mice, illustrating the anti-atherogenic properties of the enzyme.[7] Moreover, HO-1 is an important modulator of atherosclerosis, determining lesion progression and/or stabilization.[8] HO-1 has also been intensively studied in ischemia-reperfusion[9-12] and acute kidney injury models.[13] However, in the context of CKD, data on HO-1 are scarce, especially in the field of atherosclerosis.

Therefore, we aimed to characterize atherosclerotic plaque lesions of patients with peripheral artery disease with and without CKD and to study HO-1 protein and mRNA expression in these lesions. As a secondary aim, we performed immunostainings and RNA expression analysis of following markers relevant to atherosclerosis: cluster of differentiation 36 (CD36) as an important low density lipoprotein (LDL)-scavenger receptor,[14] matrix metalloproteinase2 (MMP2), involved in the remodeling process of fibrous content of plaques,[15] intercellular cell adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) as markers of endothelial dysfunction and initiation of atherosclerosis, activated caspase 3 ((a)CASP3) as marker of apoptosis and monocyte chemoattractant protein 1 (MCP1) as an important chemokine.

Concise Methods

We refer to Online Supplement 3.1 for details of the methodology.

Patients

One hundred and fifty-five patients (122 males, median age 69 [63-73]) provided informed consent to retrieve an arterial biopsy. The patients were classified as nonCKD patients (n=109) (defined as eGFR (CKD-EPI formula) >60 ml/kg/1.73m² and exclusion of structural renal damage or proteinuria) and CKD patients (n=44; 10 G3a, 14 G3b, 5 G4 , 15 G5 (of whom 10 in dialysis) according to KDIGO classification.[16] Two patients with KDIGO CKD G2 were excluded from further analysis. No other in- or exclusion criteria were used.
The study adhered to the principles of the Declaration of Helsinki and was approved by the ethics committee of the University Hospitals Leuven, Belgium.

Demographical, clinical and biochemical data

Demographical and clinical data were retrieved from the patient medical files through thorough file review (KD). For patients with CKD, primary renal diagnosis was defined according to the new ERA-EDTA Primary Renal Diagnosis (PRD) coding system.[17] Comorbidity was assessed using the method described by Charlson et al.[18] The cardiovascular risk profile was calculated based on the presence or absence of Framingham Risk factors diabetes, AHT, smoking (current or quit <5 years), obesity, dyslipidemia or positive familiar history of CVD, with a maximum score of 6.

Blood sampling and biochemical data

Blood samples were taken at the day of hospitalization or the day of surgery, before the surgical intervention for routine lab tests and further analysis on plasma and serum.

F2-isoprostanes

The total (free + esterified) concentration of the isoprostane 8-isoPGF2α (or iPF2αIII) was measured according to the methodology developed in the Laboratory of Nephrology, Department of Immunology and Microbiology, KU Leuven (HDL, unpublished).

Uremic Toxins: p-cresyl sulfate, indoxyl sulfate

P-cresyl sulfate and indoxyl sulfate were quantified as described previously,[19] using a high-performance liquid chromatography system (Alliance 2695, Waters, Belgium), coupled to a Waters 2475 fluorescence detector.

High-sensitive CRP (hsCRP) and interleukin 6 (IL-6) were measured in serum using enzyme-linked immunosorbent assays (hsCRP ELISA Demeditec, Kiel-Wellsee (Germany) and IL-6 HS ELISA e-Bioscience, Vienna (Austria)), performed according to the manufacturer's instructions.

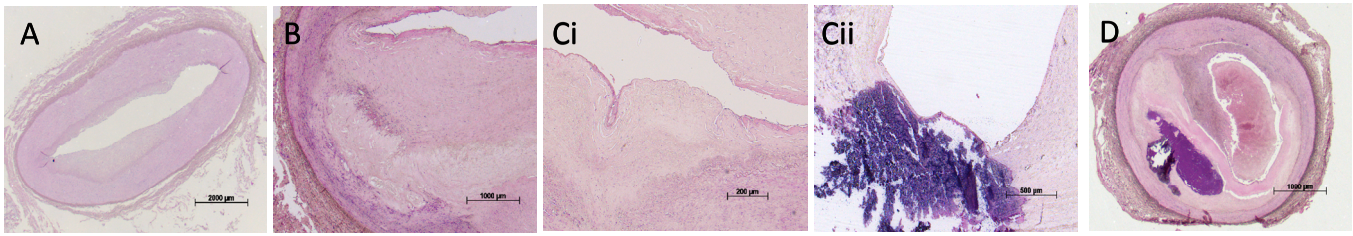
Artery sampling, histologic analyses, RNA expression analysis

Arterial biopsies procured during vascular surgery were immediately divided in three parts: one part for paraffin sections, one part for frozen sections and the third part for storage on RNAlater stabilization fluid.

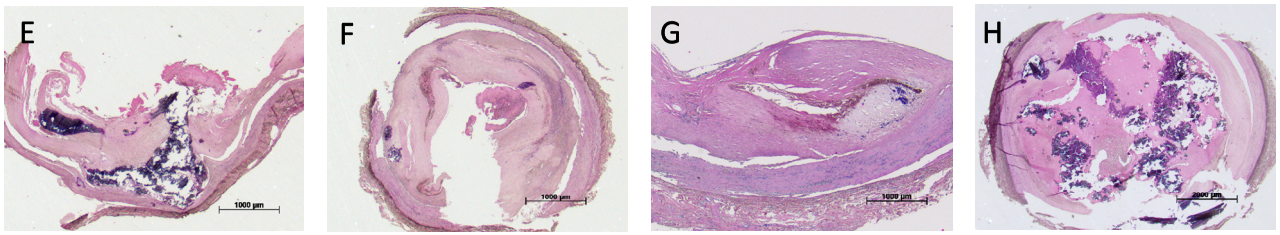
Histology and immunohistological analysis by light microscopy

Histologic analysis by Light Microscopy (LM) included 1. Identification of the type of atherosclerotic Lesions, 2. Identification of the type of complication and 3. Estimation of the evolution of the development of the former two. Lesions were defined and characterized according to the adapted AHA classification (Virmani et al.[20,21]). They are illustrated in Figures 3.1A-K. Basic, atherosclerotic lesions were classified as normal, intimal thickening (not shown), intimal xanthoma (IX, Figure 3.1A), pathological intimal thickening (not shown), fibrous cap atheroma (FCA, Figure 3.1B), Thin fibrous cap atheroma (TFCA, Figure 3.1Ci-ii) and fibrocalcific plaque (FCP, Figure 3.1D). Complications included plaque rupture (Figure 3.1E), erosion (Figure 3.1F), hemorrhage (Figure 3.1G) and total occlusion (Figure 3.1H). A calcified nodule was encountered only once (not shown). Finally, evolution and maturity were classified based on H&E and αSMA. Evolution was classified in completely organized (Figure 3.1I) and actively remodeling lesions (Figure 3.1J-K). Next, presence, location (media or (neo)intimal) and characteristics of plaque calcification was judged (spotty or nodular calcification) on the H&E sections. Fibrous cap thickness and integrity were judged on H&E and αSMA. Neovascularization was judged on H&E and αSMA by a semiquantitative scoring (0-3 with 0 'no neovascularization' to 3 'strong neovascularization). A selection of 26 sections was additionally stained for CD31 (Figure 3.4A-B). Presence of inflammatory cells was judged on the H&E staining (lymphocytes and eosinophils) and CD68 staining for mononuclear cells (macrophages and/or foam cells, score from 0-3, Figure 3.4C-D). The degree of total inflammation was calculated by the sum of scores for macrophages, foam cells and lymphocytes (0-9). Presence of iron deposition was noted (0-1). Immunohistochemistry for HO-1, MMP2, aCASP3, CD36, MCP1, ICAM1 and VCAM1 was scored in a semiquantitative cell based manner by scoring from 0 (absent) to 3 (strong staining) in each cell type. Overall lesion staining intensity was then calculated from the sum of scores in the different cell types.

Lesion Types



Complication Types



Lesion maturity

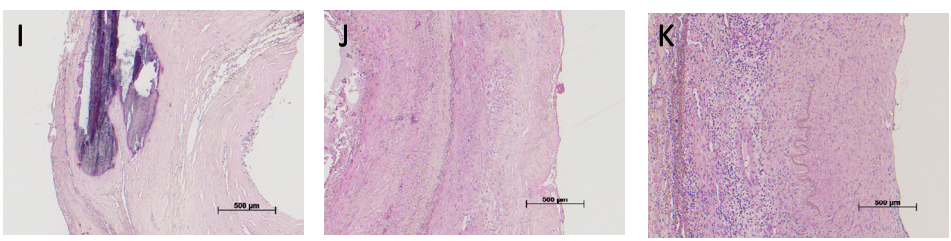


Figure 3.1 Histological classification of the atherosclerotic lesions: Light microscopic views (hematoxylin and eosin staining) of the different lesion types (A-D), lesion complications (E-H) and maturity scores of the lesions (I-K). Original magnification x12.5 (A,D,E,F and H), x25 (B and G) and x50 (Ci, Cii, I, J and K).

RNA extraction and gene expression analysis

Part of the artery biopsies was transferred into RNAlater stabilization fluid. Total RNA was extracted and resulting RNA-concentrations and purity of the RNA were determined by the use of the Nanodrop 2000c spectrophotometer. Integrity of the extracted RNA was verified using the BioAnalyzer on a random subset of 20 arteries. For the generation of cDNA 50 ng of RNA was prepared with the SuperScript® VILO™ Master Mix. Gene expression analysis was performed using a Taqman Assay for the Target genes and GAPDH as endogenous control gene. The relative expression level of the gene of interest was calculated using the $2^{-\Delta\Delta}C(_T)$ method and expressed as a Relative Quantitation (RQ=times upregulated) compared to a non-atherosclerotic nonCKD artery sample. The RQ values were categorized as follows: RQ<0.5: significant downregulation, 0.5<RQ<2.5: equal gene expression, 2.5<RQ<50: mild upregulation and RQ>50: strong upregulation of the target gene from the atherosclerotic as compared to the nonatherosclerotic control.

Statistics

Continuous variables are expressed as median and interquartile range. Categorical variables are expressed as percentages.

Comparisons between groups were made using non-parametric ANOVA (Wilcoxon Two-Sample test) for continuous data and Fisher's Exact test and Cochrane-Mantel Haenszel scores of association for categorical data. *P*-values <0.05 were considered significant.

For the multivariate analyses, missing values for PTH (n=7), serum phosphate (n=3) and albumin (n=3) were replaced by their respective mean values from either the nonCKD or CKD group. For the CKDG5D patients, eGFR was arbitrarily set on 5 ml/min/1.73m² (n=6).

Based on the findings of the comparative analyses, multivariate logistic regression analysis was performed to define variables associated with plaque complications.

Furthermore, determinants of neovascularization and HO-1 expression were explored in detail using univariate Spearman Rank correlation statistics, followed by multivariate linear regression analysis.

The SAS (version 9.3, the SAS institute, Cary, N.C., USA) software package was used for the statistical analyses.

Results

Patients, demographical, clinical and biochemical data

Table 3.1 shows demographical, clinical and biochemical characteristics of the 153 patients included in the analysis.

Table 3.1 Demographic and clinical data of all included patients.

153	Non-CKD	CKD 3-5D		N 109/44
Male %	82	71	0.13	109/44
Age	67 [62-72]	71 [67.5-76]	0.002	109/44
eGFR ml/min/1.73m²	79 [67-90]	31 [13-43]	< 0.0001	109/44
Hypertension %	78	89	0.17	109/44
Diabetes %	15	34	0.01	109/44
Dyslipidemia %	85	84	0.80	107/44
BMI kg/m²	26.4 [23.4-29.6]	24.5 [22.0-28.2]	0.05	109/44
Smoking				109/44
Active %	42	30	0.42	
Former %	52	61		
Never %	6	9		
Pack years	30 [25-45]	35[15-46]	0.90	95/37
Lipid lowering drugs %	75	68	0.42	109/44
Antihypertensives %	78	93	0.03	109/44
CVRF /6	3 [2-4]	3 [2-4]	0.51	109/44
Charlson score	4 [3-6]	7 [6-8.5]	< 0.0001	108/44
CAD %	44	61	0.05	109/44
AMI %	23	43	0.04	106/44
Carotid disease %	38	55	0.01	108/41
stroke %	10	14	0.57	109/44
Type of surgery				
AAA	17	23	0.52	109/44
PAD intervention, nr	1 [0-3]	0 [0-2]	0.37	109/44
Complicated PAD %	29	18	0.23	109/44
CVD %			0.13	
1	42	27		
2	68	73		
Bio-Intact PTH (ng/L)	22.0 [15.1-31.0]	37.1 [20.6-107.8]	< 0.0001	94/42
25-OH-Vitamin D (µg/L)	28 [16.3-37.3]	31.4 [18.9-42.5]	0.24	99/41
Cholesterol (mg/dL)	159.5 [135.0-190.0]	148.0 [126.5-181]	0.13	106/44
LDL (mg/dL)	78.0 [63.0-97.0]	74.0 [56.0-102.0]	0.51	105/44
HDL (mg/dL)	48.5 [39.0-59.0]	41.0 [35.0-51.0]	0.02	106/44
Triglycerides (mg/dL)	132.0 [96.0-184.0]	126.0 [102.5-176.5]	0.94	106/44
CRP (mg/L)	3.2 [0.8-0.1]	3.2 [0-12.3]	0.74	92/41
phosphate (mg/dL)	3.2 [2.9-3.5]	3.4 [2.8-3.8]	0.05	99/43
bicarbonate (mmol/dL)	25.8 [24.0-27.2]	25.1 [22.4-26.7]	0.06	108/44
Hemoglobin (g/dL)	13.7 [12.7-14.7]	11.8 [10.1-13.7]	< 0.0001	108/44
Albumin (g/L)	44.8 [42.6-46.8]	41.8 [38.5-46.2]	0.02	100/43
IL-6 (pg/mL)	2.86 [1.49-6.88]	2.78 [1.55-5.74]	0.64	98/40
F2-isoprostanes (pg/mL)	90.0 [70.0-133.0]	107.0 [59.0-140.0]	0.69	101/38
hsCRP (µg/mL)	2.66 [0.98-7.36]	5.04 [1.77-12.95]	0.03	105/42
P-cresyl sulfate (µM)	24.2 [12.5-43.4]	40.6 [28.3-93.7]	< 0.0001	106/41
Indoxyl sulfate (µM)	4.7 [3.1-7.0]	9.4 [5.8-21.3]	< 0.0001	106/41

AMI acute myocardial infarction, BMI body mass index, CAD coronary artery disease, CKD chronic kidney disease, CRP C-reactive protein, CVD cardiovascular disease, CVRF cardiovascular risk factors, eGFR estimated glomerular filtration rate, FCA fibrous cap atheroma, FCP Fibrocalcific plaque, HDL High-density lipoprotein, hsCRP high sensitive C-reactive protein, LDL low-density lipoprotein, n Number, PAD peripheral artery disease, PTH parathyroid hormone.

CKD patients have lower eGFR, higher proportion of diabetes, higher PTH, phosphate, p-cresyl sulfate and indoxyl sulfate and lower serum albumin, bicarbonate and hemoglobin than nonCKD patients. Furthermore, they are older, have a lower BMI and use more anti-hypertensives. Although not different from nonCKD patients with regard to peripheral artery disease, more CKD patients have a history of coronary artery disease, acute myocardial infarction and carotid disease. Finally, they have lower HDL cholesterol and higher hsCRP. There is no difference between the two groups in IL-6 or F2-isoprostanes.

Despite their consent for artery biopsy, in only 86 of the 153 patients arterial lesion biopsies were available for histological phenotyping. Indeed, patient safety rendered biopsy procurement impossible in 67 patients.

Table 3.2 shows demographical, clinical and biochemical characteristics of the 41 nonCKD and 25 CKD patients (4 G3a, 8 G3b, 4 G4, 3 G5 and 6 G5D) with these lesion types. Causes of renal disease of the CKD patients in this subgroup are systemic disease affecting the kidney [diabetic nephropathy (n=3), renal vascular disease (n=9)], familial/hereditary kidney disease [polycystic kidney disease (n=1)], glomerular disease (n=2), miscellaneous (n=3) and unknown etiologies (n=7). Differences between nonCKD and CKD within this subgroup are essentially the same as in the entire cohort. Some differences lose significance however (see Table 3.2).

Atherosclerotic lesions: general pathology

Morphology

Of the 86 biopsies available for histological phenotyping of the lesions, 16 biopsies are found to be non-interpretable because of low quality of the procured material. Four arterial biopsies show no or only minor abnormalities. Thirteen biopsies show FCA and 53 FCP lesions. These 66 biopsies allow further detailed lesion characterization (See Flow Chart in the supplemental files).

Based on morphological evaluation on H&E and αSMA sections, 74% of the 66 FCA/FCP lesions are organized lesions and 26% remodeling lesions. Eighty-eight % contain plaque calcifications, with a majority of nodular calcifications (81%). The fibrous cap (not evaluable in 8/66 sections) is thin in 25% and interrupted in 35% of the plaques. Complications are found in 41% of the lesions. The degree of neovascularization does not differ between FCA and FCP. Inflammatory cells in the plaques consist mainly of macrophages, foam cells and to a lesser extent lymphocytes.

See Online Supplement 3.2 for general description of immunohistochemistry and gene expression analysis.

Table 3.2 Demographic and clinical data of the biopsy groups.

FCA/FCP n=66	Non-CKD	CKD 3-5D		N 41/25
Male %	93	80	0.126	41/25
Age	68 [62-73]	72 [69-76]	0.05	41/25
eGFR ml/min/1.73m²	84 [71-97]	31 [26-42]	< 0.0001	41/19
Hypertension %	83	92	0.297	41/25
Diabetes %	15	32	0.094	41/25
Dyslipidemia %	95	80	0.043	41/25
BMI kg/m²	24.3 [21.7-27.8]	25.8 [23.4-28.4]	0.056	41/25
Smoking				41/25
Active %	39	36	0.623	
Former %	51	60		
Never %	10	4		
Pack years	30 [25-40]	35 [30-40]	0.37	34/21
Lipid lowering drugs %	78	68	0.365	41/25
Antihypertensives %	90	92	0.809	41/25
CVRF /6	3 [3-4]	3 [2-4]	0.38	41/25
Charlson Score	7 [6-9]	4 [3-6]	< 0.0001	41/25
CAD%	49	64	0.23	38/24
AMI %	27	48	0.14	39/25
Carotid disease %	39	60	0.092	40/23
Stroke %	15	20	0.570	41/25
Type of surgery				
AAA %	27	32	0.781	41/25
PAD intervention, n	1 [0-2]	1 [0-2]	0.47	41/25
Complicated PAD %	25	24	0.97	41/25
CVD %				41/25
1	33	12	0.09	
2	66	76		
Bio-intact PTH (ng/L)	19.9 [13.8-31.0]	40.3 [29.2-85.5]	0.0005	35/24
25-OH-Vitamin D (µg/L)	25 [16.7-36.0]	30.3 [19.7-42.5]	0.1974	35/24
Cholesterol (mg/dL)	162 [135-193]	147 [124-177]	0.32	41/25
LDL (mg/dL)	78 [62-108]	72 [55-101]	0.35	41/25
HDL (mg/dL)	44 [38-56]	45 [39-51]	0.92	38/25
Triglycerides (mg/dL)	138.5 [97.5-200.5]	130 [89-177]	0.44	40/25
CRP (mg/L)	3.0 [0.7-6.6]	2.2 [0.1-8.0]	0.51	37/25
Phosphate (mg/dL)	3.3 [2.8-3.5]	3.5 [3.1-3.9]	0.07	38/25
Bicarbonate (mmol/dL)	25.8 [24.2-26.9]	24.6 [21.8-25.9]	0.03	41/25
Hemoglobin (g/dL)	13.3 [12.9-14.3]	11.8 [10.4-13.2]	0.001	41/25
Albumin (g/L)	45.2 [43.1-47.3]	44.0 [39.4-47.3]	0.26	38/25
Interleukin 6 (pg/mL)	3.1 [1.4-6.4]	3.2 [2.2-7.4]	0.1659	41/25
F2-isoprostanes (pg/mL)	8.0 [71.0-133]	115 [59-140]	0.72	39/21
hsCRP (µg/mL)	3.7 [1.0-7.4]	4.2 [1.8-8.9]	0.34	41/25
P-cresyl sulfate (µM)	27.6 [11.6-48.0]	47.5 [30.6-96.3]	< 0.0001	41/25
Indoxyl Sulfate (µM)	4.5 [3.0-7.2]	14.4 [6.9-28.3]	< 0.0001	41/25

AMI acute myocardial infarction, BMI body mass index, CAD coronary artery disease, CKD chronic kidney disease, CRP C-reactive protein, CVD cardiovascular disease, CVRF cardiovascular risk factors, eGFR estimated glomerular filtration rate, FCA fibrous cap atheroma, FCP Fibrocalcific plaque, HDL High-density lipoprotein, hsCRP high sensitive C-reactive protein, LDL low-density lipoprotein, n Number, PAD peripheral artery disease, PTH parathyroid hormone.

Organized versus remodeling atherosclerotic lesions

Table 3.3 shows plaque characteristics according to maturity of the FCA/FCP lesions. No difference between organized and remodeling lesions is found regarding lesion type, plaque calcification, cap characteristics and neovascularization. However, remodeling lesions display more plaque complications (65 vs. 35 %, *P*=0.048) and are characterized by higher numbers of foam cells, lymphocytes and macrophages (median total inflammation score 4.5 vs. 3.0, *P*=0.05).
Overall immunohistochemical staining (Overall IHC) for MMP2, MCP1, CD36, VCAM1 and ICAM1 is significantly stronger in remodeling lesions as compared to organized lesions (Figure 3.2 *P*=0.01; 0.02; 0.02; 0.01 and 0.007 respectively). On gene expression level, as compared to organized lesions, remodeling lesions show more downregulated CASP3 (29% vs. 8.5%, *P*=0.0482) (Figure 3.3).

Table 3.3 Lesion Characterization Organized vs Remodeling lesions.

		Organized (49)	Remodeling (17)	Fisher's Exact	N
Lesion type	FCA %	15	24	0.3949	49/17
	FCP %	85	76		
Lesions characteristics	Plaque Calcification %	88	88	0.8775	49/17
	Spotty %	7	6		
	Nodular %	81	82		
Fibrous cap	Interrupted %	26	56	0.0612	42/16
Complicated lesion %		35	65	0.048	48/17
Complication type	Calcified nodule %	6	0		
	Erosion %	35	45		
	Hemorrhage %	12	0		
	Plaque rupture %	29	45		
	Occlusion %	18	9		
		Median (range)	Median (range)		
Neovascularization		1.0 [0.5-2.0]	1.0 [1.0-2.0]	0.9938	48/17
Inflammatory cells	Macrophages	1.0 [1.0-2.0]	1.0 [1.0-2.0]	0.0715	48/17
	Foam cells	2.0 [1.0-3.0]	2.0 [2.0-3.0]	0.1113	
	Lymfocytes	0 [0.0-1.0]	1.0 [0.5-1.0]	0.0681	
	sum inflammation	3.0 [2.0-5.0]	4.5 [3.0-6.0]	0.0528	

FCA fibrous cap atheroma, FCP Fibrocalcific plaque, n Number.

Lesions from nonCKD versus CKD patients

Table 3.4 shows morphology and immunohistochemistry data of the FCA/FCP lesions according to CKD category. No difference between nonCKD and CKD patients is found with regard to lesion type, maturity and plaque calcification. However, a higher proportion of the fibrous caps is interrupted (57% vs. 22%, *P*=0.0077) and there are more plaque complications (68% vs. 27%, *P*=0.0018) in the CKD group. Lesions from CKD patients are characterized by higher numbers of foam cells, lymphocytes and macrophages (median total inflammation score 5.00 vs. 3.00, *P*=0.04, Figure 3.4C-D).

The level of neovascularization is highest in CKD patients (median score 2.00 vs. 1.00, *P*=0.04, Figure 3.4A-B).
Overall IHC for MCP1 and aCASP3 is significantly stronger in lesions from CKD patients as compared to nonCKD patients (Figure 3.5 *P*=0.04 and 0.038 respectively). On gene expression level, nonCKD lesions show clearly more often upregulated HO-1 as compared to CKD lesions (92.5% vs. 64% *P*=0.0029) (Figure 3.6).

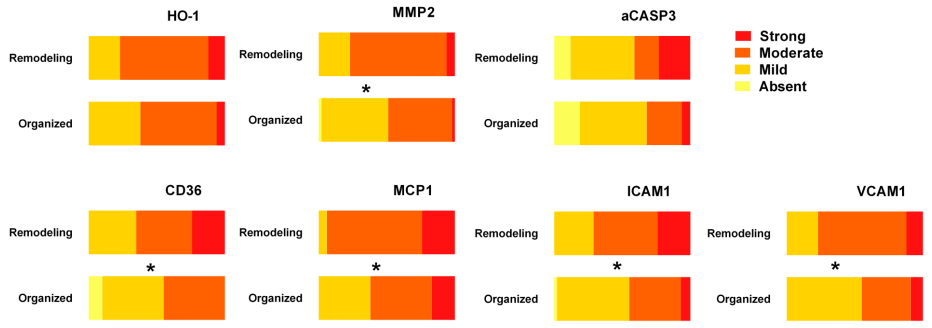


Figure 3.2 Immunohistochemistry of atherosclerotic arteries in organized versus remodeling lesions. Semiquantitative scores for overall HO-1, MMP2, aCASP3, CD36, MCP1, ICAM1 and VCAM1 intensity in the FCA/FCP lesions. Score range from 0 (light yellow: absent staining in cells) to 3 (red: strong intensity of staining in cells). The fractions of lesions with the defined intensity are given. * *P*-value < 0.05.

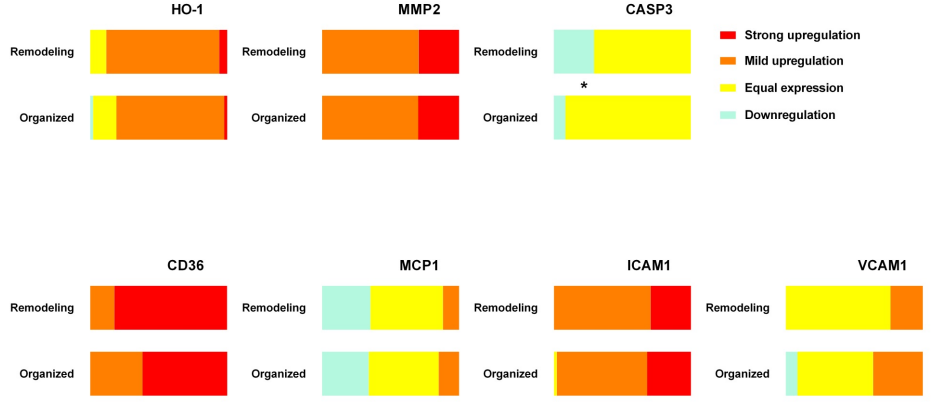


Figure 3.3 RNA expression of atherosclerotic arteries in organized versus remodeling lesions. Relative quantification for HO-1, MMP2, CASP3, CD36, MCP1, ICAM1 and VCAM1 intensity in the nonCKD and CKD FCA/FCP lesions as referenced against a non-atherosclerotic nonCKD artery specimen. RQ values were categorized into a score range as follows: Downregulated (RQ <0.5 blue), equal expression 0.5<RQ<2.5 yellow), mild upregulation (2.5<RQ<50), strong upregulation(RQ>50; red). The fractions of arteries with the defined intensity are given as parts of a whole. * *P*-value <0.05.

Table 3.4 Lesions characteristics nonCKD versus CKD.

		Non-CKD	CKD 3-5D	P	N
Surgery type	AAA %	27	30		41/25
Lesion type	FCA %	21	15		41/25
	FCP %	74	78	0.55	
Lesions characteristics	Organised lesion %	76	72	0.71	40/25
	Plaque Calcification %	85	92	0.42	
	Spotty %	10	4		
	Nodular %	75	88		
Fibrous cap	Interrupted %	22	57	0.0077	36/21
Complicated lesion %		27	68	0.0018	41/25
Complication types	Calcified nodule %	0	1		
	Erosion %	4	7		
	Hemorrhage %	0	2		
	Plaque rupture %	5	5		
	Occlusion %	2	2		
		Median (range)	Median (range)		
Neovascularization		1.0 [0.5-2.0]	2.0 [1.0-2.0]	0.04	41/25
Inflammatory cells	Macrophages /3	1.0 [1.0-2.0]	1.0 [1.0-2.0]	0.256	
	Foam cells /3	1.0 [1.0-2.0]	2.0 [2.0-3.0]	0.004	
	Lymfocytes /3	0.5 [0.0-1.0]	0.5 [0.0-2.0]	0.75	
	Total inflammation /9	3.0 [2.0-5.0]	5.0 [3.0-6.0]	0.04	

FCA fibrous cap atheroma, FCP Fibrocalcific plaque, n Number.

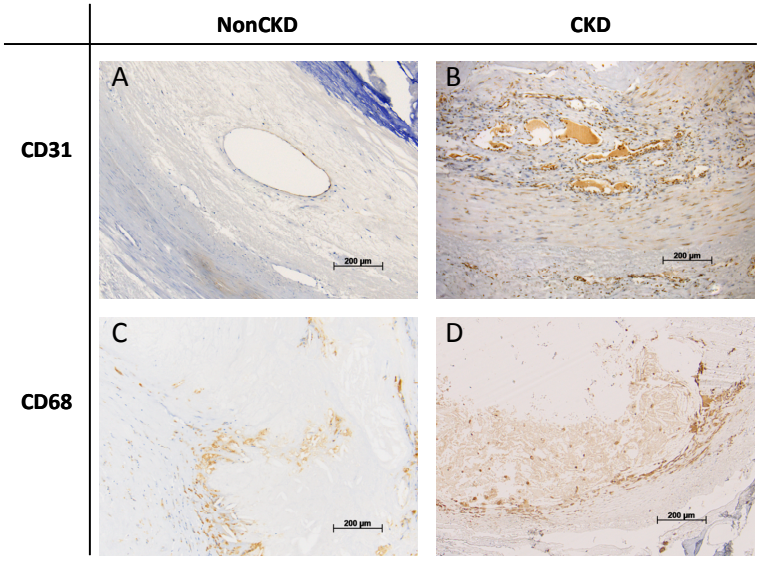


Figure 3.4 Mononuclear cell infiltration and neovascularization in lesions of nonCKD vs CKD patients. Light microscopic view of fibrocalcific plaques of a nonCKD (A and C) and a CKD (B and D) patient. (A-B) representative pictures showing immunoreactivity for CD31, reflecting neovascularization. (C-D) representative pictures showing immunoreactivity for CD68, reflecting infiltration by macrophages and foam cells. Original magnification x100.

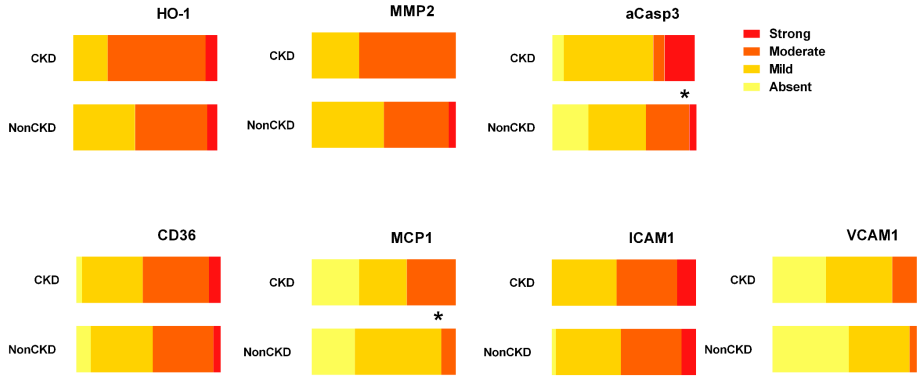


Figure 3.5 Immunohistochemistry of atherosclerotic arteries in nonCKD versus CKD. Semiquantitative scores for overall HO-1, MMP2, aCASP3, CD36, MCP1, ICAM1 and VCAM1 intensity in the nonCKD and CKD FCA/FCP lesions. Score range from 0 (light yellow: absent staining in cells) to 3 (red: strong intensity of staining in cells). The fractions of lesions with the defined intensity are given. * *P*-value <0.05.

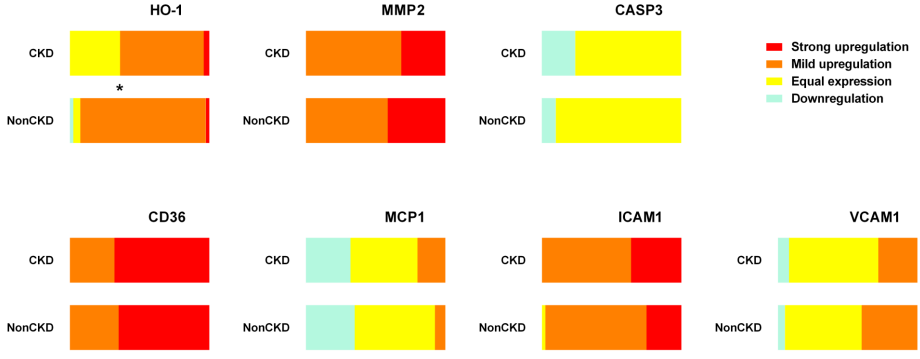


Figure 3.6 RNA expression of atherosclerotic arteries in nonCKD versus CKD. Relative quantification for HO-1, MMP2, CASP3, CD36, MCP1, ICAM1 and VCAM1 intensity in the nonCKD and CKD FCA/FCP lesions as referenced against a non-atherosclerotic nonCKD artery specimen. RQ values were categorized into a score range as follows: Downregulated (RQ <0.5 blue), equal expression 0.5<RQ<2.5 yellow), mild upregulation (2.5<RQ<50), strong upregulation(RQ>50; red). The fractions of arteries with the defined intensity are given as parts of a whole. * *P*-value < 0.05.

Integrating lesion maturity and CKD status

Figure 3.7 integrates the central finding of the results of Tables 3.3 and 3.4. While the proportion of organized lesions in the nonCKD and CKD group is equal (76 vs. 72%, see Table 3.4), the CKD lesions show higher rates of plaque complications and higher inflammation scores. As such, despite their advanced morphological status these

organized lesions of CKD patients continue to express the characteristics of "remodeling" atherosclerotic lesions. These characteristics, together with high levels of neovascularization, render the plaques of CKD patients more vulnerable than those of their nonCKD counterparts.

Determinants of plaque complications, neovascularization and HO-1 expression

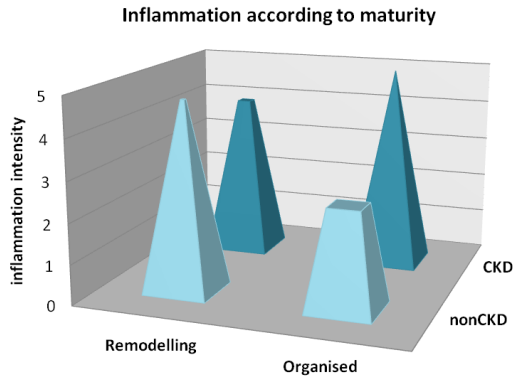
The vulnerable plaque phenotype of CKD patients is further explored by studying determinants of plaque complications, neovascularization and HO-1 given their role in plaque vulnerability and as an important plaque-stabilizing enzyme, respectively.

Univariate correlation analysis

The presence of plaque complications is positively associated with Charlson Score, hsCRP, total plaque inflammation, overall IHC for HO-1, ICAM1 and MMP2 as well as with the levels of calcification and neovascularization of the plaque. A significant inverse correlation is noted with eGFR, hemoglobin and serum albumin (Table 3.5).

Neovascularization correlates positively with Charlson Score, hsCRP, total plaque inflammation, the presence of plaque complications, overall IHC for HO-1 and CD36, and gene expression of CASP3. A significant inverse correlation is noted for hemoglobin and eGFR (Table 3.5).

The overall IHC for HO-1 correlates significantly with Charlson Score, total plaque inflammation, overall IHC for MMP2, aCASP3, CD36, the presence of plaque complications and the level of neovascularization.



	Remodelling	Organised
nonCKD	4,7	2,5
CKD	4	5

Figure 3.7 Integrating organized vs Remodeling and nonCKD vs CKD lesions. 3D chart showing the distribution of inflammatory highly active lesions according to maturity of the lesion or presence of CKD. The median inflammatory activity is given with a score of 0-12.

Table 3.5 Univariate associations: Plaque Complications, Neovascularization, overall IHC for HO-1 P values < 0.05 are bolded. * other *P* values <0.2.

	Plaque Complications		Neovascularization		IHC heme oxygenase-1	
	Estimate	p-value	Corr Coeff	p-value	Corr Coeff	p-value
Demographic						
Age	0.0446	0.2034	0.0792	0.5241	0.0649	0.6018
Sex	-0.1886	0.6173	-0.016	0.896	0.12664	0.3072
BMI	-0.0239	0.6966	0.234	0.057*	0.14213	0.2513
Diabetes	0.4212	0.4854	0.162	0.191*	0.02954	0.8124
Charlson score	**0.3485**	**0.0064**	**0.329**	**0.0014**	**0.25339**	**0.0386**
Biochemical						
eGFR	**-0.0228**	**0.0059**	**-0.248**	**0.042**	-0.16519	0.1816*
Ln (Bio-Intact PTH)	0.3944	0.1971*	0.175	0.157*	0.11153	0.3689
Ln (HDL)	-0.0202	0.2902	-0.159	0.199*	-0.10723	0.3877
Hemoglobin	**-0.2770**	**0.0456**	**-0.276**	**0.024**	-0.12417	0.3168
Ln (hsCRP)	**0.6406**	**0.0161**	**0.302**	**0.013**	0.21575	0.0795*
Ln (Interleukin 6)	0.4766	0.0814*	0.160	0.197*	0.16721	0.1762*
Ln (Albumin)	**-0.1284**	**0.0321**	-0.11608	0.3496	-0.11492	0.3544
Ln (Indoxyl Sulphate)	0.2813	0.1756*	0.13235	0.2894	0.01402	0.9110
Ln (F2-Isoprostanes)	-0.6430	0.2208	-0.10896	0.4073	-0.16067	0.2201
Morphological – Immunohistochemistry						
Plaque inflammation	**0.5610**	**0.0006**	**0.565**	**<0.0001**	**0.36003**	**0.0028**
Overall IHC HO-1	**0.3810**	**0.0025**	**0.392**	**0.001**	**-**	-
Overall IHC MMP2	**0.2984**	**0.0035**	0.223	0.074*	**0.27574**	**0.0262**
Overall IHC aCASP3	0.2907	0.0642*	0.177	0.157*	**0.27105**	**0.0295**
Overall IHC CD36	0.0954	0.2214	**0.326**	**0.008**	**0.24925**	**0.0453**
Overall IHC ICAM1	**0.2749**	**0.0084**	0.158	0.208	0.16848	0.1797*
Overall IHC VCAM1	0.1679	0.3468	0.162	0.197*	0.19224	0.1250*
Overall IHC MCP1	0.0305	0.8292	0.05517	0.6625	0.04794	0.7045
Neovascularization	**1.1386**	**0.0002**	-	-	**0.39234**	**0.001**
Calcification	**1.088**	**0.0421**	-0.06905	0.5787	0.10804	0.3842
Plaque complication	-	-	**0.38147**	**0.0014**	**0.38566**	**0.0013**
RNA gene expression						
HO-1	0.4699	0.3919	0.01499	0.9049	0.11465	0.3593
MMP2	0.2824	0.6058	-0.09188	0.4631	0.13509	0.2795
CASP3	0.1178	0.8663	**0.293**	**0.017**	0.21390	0.0846*
CD36	0.6711	0.22	0.187	0.131*	0.11564	0.3552
ICAM1	0.5117	0.3219	0.11255	0.3683	0.12777	0.3066
VCAM1	-0.5943	0.1873*	-0.03768	0.7639	-0.10526	0.4003
MCP1	0.3645	0.3426	0.18741	0.1319	0.02763	0.8257

(a)CASP3 (activated) caspase-3, BMI body mass index, CD36 cluster of differentiation 36, Corr coeff correlation coefficient, CRP C-reactive protein, eGFR estimated glomerular filtration rate, HDL High-density lipoprotein, HO-1 heme oxygenase-1, hsCRP high sensitive C-reactive protein, ICAM1 intercellular adhesion molecule 1, LDL low-density lipoprotein, Ln logaritmus naturalis, MCP1 monocyte chemoattractant protein 1, MMP2 matrix metalloproteinase 2, n Number, PTH parathyroid hormone, VCAM1 vascular cellular adhesion molecule.

Multivariate logistic regression analysis for plaque complications

Table 3.6 shows the final model resulting from backward selection using the best subset of variables which includes eGFR, neovascularization, calcification, overall IHC HO-1, overall IHC MMP2, overall IHC ICAM1, ln hsCRP. Neovascularization, eGFR

(MDRD), calcification, overall IHC MMP2, and overall IHC ICAM1 are found to be independently associated with plaque complications.

Table 3.6 Logistic regression analysis for plaque complications.

Plaque Complication
Selected subset after initial screening:
eGFR (MDRD), Neovascularization, Calcification, overall IHC HO-1, overall IHC MMP2, overall IHC ICAM1, ln (hsCRP)
Variables consecutively removed from the model by backward elimination (p-value at elimination):
overall IHC HO-1 (0.1625), ln (hsCRP) (0.1366)
Final model: Backward elimination

Variable	BETA	Unit of increase	OR	95% CI	p-value
eGFR (EPI)	-0.0284	1 ml/min/1.73m²	0.972	0.952-0.993	0.0088
Neovascularization	0.9929	1 unit	2.699	1.015-7.174	0.0465
overall IHC ICAM1	0.3263	1 unit	1.386	1.003-1.914	0.0476
Overall IHC MMP2	0.3036	1 unit	1.355	1.011-1.815	0.0419
Calcification	1.752	1 unit	5.770	1.307-25.472	0.0207

CI confidence interval, eGFR estimated glomerular filtration rate, HO-1 heme oxygenase-1, hsCRP high-sensitive C-Reactive protein, ICAM1 intercellular adhesion molecule 1, IHC immunohistochemical staining, Ln logaritmus naturalis, MMP2 matrix metalloproteinase 2, OR Odds Ratio.

Multivariate linear regression analyses for neovascularization and HO-1 staining

In Tables 3.7 and 3.8, the final models explaining the variability in neovascularization and overall HO-1 staining score following the methodology defined in the methods section are shown. Variables resulting from the best subset selection step for neovascularization were eGFR, diabetes, total plaque inflammation and overall IHC for aCASP3, VCAM1 and HO-1. Total plaque inflammation and eGFR are found to be independently associated with neovascularization (Table 3.7).

Table 3.7 Multivariate backward linear regression analysis for neovascularization.

Neovascularization
Selected subset after initial screening:
eGFR, Diabetes 0/1, plaque inflammation, Overall IHC aCASP3, Overall IHC VCAM1, Overall IHC HO-1.
Variables consecutively removed from the model by backward elimination (p-value at elimination):
Overall IHC aCASP3 (0.1437), Overall IHC HO-1 (0.1534), Diabetes 0/1 (0.0973), Overall IHC VCAM1 (0.0733)
Final Model Backward elimination R^2 0.3656

Variable	BETA	P-value
eGFR (EPI)	-0.00567	0.0206
Plaque Inflammation	0.21998	<0.0001

aCASP3 activated caspase-3, eGFR estimated glomerular filtration rate, HO-1 heme oxygenase-1, IHC immunohistochemical staining, VCAM1 vascular cell adhesion molecule 1.

Variables resulting from the best subset selection step for overall HO-1 staining were neovascularization, total plaque inflammation, serum HCO3 and overall IHC for aCASP3, MMP2 and MCP1. Total plaque inflammation, serum HCO3 and overall staining for aCASP3 and MCP1 are found to be independently associated with the intensity of HO-1 staining in the plaque (Table 3.8).

Table 3.8 Multivariate backward linear regression analysis for HO-1

Overall IHC Heme oxygenase-1		
Selected subset after initial screening:		
Plaque inflammation, Overall IHC MMP2, Overall IHC aCASP, overall IHC MCP1, bicarbonate, Neovascularization		
Variables consecutively removed from the model by backward elimination (p-value at elimination):		
overall IHC MMP2 (0.0920), angiogenesis (0.1899)		
Final Model Backward elimination R^2 0.2806		
Variable	BETA	P-value
Plaque Inflammation	0.56716	0.0011
Overall IHC aCASP	0.52974	0.0096
Overall IHC MCP1	-0.49950	0.0108
HCO3	-0.26998	0.0358

aCASP activated caspase-3, MCP1 monocyte chemoattractant protein 1, MMP2 matrix metalloproteinase 2.

Discussion

The central findings of our study are that atherosclerotic lesions in CKD patients are characterized by higher inflammatory cell infiltration, more neovascularization and a higher proportion of plaque complications than those of nonCKD patients. In agreement, eGFR was found to be an important predictor of both intra-plaque neovascularization and atherosclerotic plaque complications, independent of traditional cardiovascular risk factors. Second, CKD lesions had a tendency to lower HO-1 expression.

The two main objectives of our study were to morphologically characterize atherosclerotic plaques and to evaluate the expression of the anti-atherogenic enzyme HO-1 in patients with CKD.

Our analysis shows no differences between CKD and nonCKD patients with regard to lesion type and plaque calcifications. However, atherosclerotic lesions of CKD patients showed a higher proportion of thin fibrous caps, and more neovascularization and inflammation. From this it is not unexpected that the proportion of plaque complications was highest in the lesions of CKD patients. Interestingly, there was no difference between CKD and nonCKD patients in the proportions of organized vs. remodeling lesions. However, by higher inflammation and neovascularization, even the morphologically organized lesions of CKD patients retain a "remodeling phenotype", as illustrated in Figure 3.7. These findings all fit within a 'vulnerable plaque'-phenotype as previously defined by several authors.[20,22-24] Indeed, a thin fibrous cap, high infiltration by (foamy) macrophages, a lipid-rich necrotic core and high levels of neovascularization are characteristics of plaques with high risk for rupture or thrombosis. Our findings are in agreement with those of Nakano et al.[25] who studied post-mortem coronary artery lesions of 126 subjects. They found significantly increased neovascularization and intraplaque complications in patients with an eGFR < 30 ml/min/1.73m² as compared to

those with an eGFR ≥60 ml/min/1.73m². In a study by Pelisek et al. in 46 CKD patients with advanced carotid stenosis, carotid plaques were more unstable (83 vs. 52%) and ruptured (59 vs. 36%) than those of a parallel series of patients with normal renal function and similarly occlusive carotid lesions.[26] The fibrous component of the plaque was markedly reduced in the CKD patients (40 vs. 57%), suggesting that this alteration may be a relevant driver of plaque instability and rupture in this condition. Indeed, next to eGFR and neovascularization, intraplaque MMP2, responsible for extracellular matrix collagen digestion and plaque remodeling, was an independent predictor in our multivariate regression model of plaque complications. Traditional risk factors of the vulnerable plaque are high total cholesterol, low HDL and high hsCRP levels.[20] In contrast to general population findings, we were not able to show an effect of lipid levels on atherosclerotic plaque characteristics. However, univariate correlation analysis in the nonCKD population confirmed the above-mentioned negative association of HDL-cholesterol with neovascularization, while this was not the case in the CKD patients (data not shown). It is tempting to speculate that in CKD patients, other factors related to the uremic state overwhelm the effects of lipids on vulnerable plaque development.[27-30] The exact CKD-related mechanisms are a matter of ongoing debate and research. Nakano et al showed that neovascularization coincided with increased expression of vascular endothelial growth factor (VEGF) in the coronary plaques of CKD patients.[25] VEGF has a pivotal role in angiogenesis.[31] The expression of VEGF is regulated mainly by hypoxia,[31] but also inflammatory mediators and Reactive Oxygen Species (ROS) are known inducers of the VEGF pathway (reviewed by Jeney et al.[32]). In our analysis, we found neovascularization to multivariately correlate with inflammation, but also univariately with overall staining for CD36 and hemoglobin. Although we have no data on VEGF expression, this finding supports the importance of VEGF-inducing conditions such as inflammation and hypoxia. Indeed, anemia as one of the frequent complications of CKD, could contribute to intraplaque hypoxia. CD36 is a pleomorphic molecule which functions as a B-scavenger receptor for oxidized low-density lipoprotein (oxLDL) and the interaction with oxLDL triggers signaling cascades for inflammatory responses.[14] At the same time, CD36 both mediates the anti-angiogenic effect of thrombospondin-1[33] and participates in plaque maturation through its interaction with thrombospondin-1 to initiate phagocytosis.[34] It is of note that we found significantly more CD36 protein in remodeling as compared to organized lesions, most probably reflecting lipid uptake by foamy macrophages and plaque organization.

Immunohistochemical HO-1 expression is determined by inflammation, apoptosis and MCP1. In univariate analysis, it is also associated with neovascularization in our study cohort. This is not unexpected since both plaque inflammation and plaque stability are influenced by HO-1, as demonstrated earlier.[8,35,36] It is important to keep in mind the stress-responsive nature of HO-1: the enzyme is induced by inflammation, tissue damage and oxidative stress and its activity results in atheroprotection in early stages of atherosclerosis, while it leads to plaque stabilization in the established

atherosclerotic plaque at a later stage. In agreement, Cheng et al showed that HO-1 expression is upregulated in human vulnerable plaques and HO-1 overexpression in apoE^{-}/$^{-}$ mice impeded atherosclerotic lesion progression into a vulnerable plaque.[8] Another study by Tingting et al confirmed these results in a vulnerable plaque model in rabbits and revealed important inhibitory effects of HO-1 on the induction and expression of matrix metalloproteinases, IL-6 and TNFα.[37]

Whether HO-1 enzyme activity is one of the factors explaining differences in neovascularization between CKD and nonCKD patients, cannot be unequivocally judged from our data. However, immunohistochemical HO-1 intensity was not significantly different between the nonCKD and CKD groups. Given the stress-responsive nature of HO-1 expression in atherosclerosis and the abovementioned association with inflammation and MCP1 in our study cohort, however, one would have expected higher expression in the more inflammatory CKD lesions. mRNA expression analysis, which is a more sensitive and accurate methodology than the semi-quantitative immuno-histochemical scoring, even showed a significantly lower proportion of *HO-1* mRNA upregulation in the CKD as compared to the nonCKD group. These data suggest that insufficient HO-1 activity in CKD patients may contribute to the higher level of inflammation, neovascularization and plaque complications seen in the atherosclerotic plaques of these patients.

The chemokine MCP1 leads to the sequestration and differentiation of mononuclear cells into (foamy) macrophages and feeds the atherogenic process. MCP1 expression is inhibited by HO-1 activity.[38] MCP1 staining was stronger in the CKD lesions. This is in agreement with the findings of higher inflammation and adds to the thesis of higher oxidative and inflammatory stress in CKD. Taken together with the HO-1 findings described above, the negative association between MCP1 and HO-1 staining support the idea of relatively impaired stress-response of HO-1 in CKD.

Caspase-3 was studied as a marker of apoptosis. It has been described as an important mediator early in the atherosclerotic pathway, linked to DNA damage.[39] Apoptosis in endothelial cells, macrophages and VSMC, however, is implicated not only in cell responses to acute stress and atherosclerosis, but also in cell homeostasis and vessel remodeling. Given this multifaceted role of apoptosis in the vessel wall and considering that CASP3 is just one of many pro-apoptotic mediators, the interpretation of its expression data in our study is complicated. We found RNA expression of the 32kd uncleaved proenzyme *CASP3* in atherosclerotic lesions to be not significantly different from the non-atherosclerotic nonCKD reference sample (data not shown). Organized lesions showed higher expression of the enzyme as compared to remodeling lesions (Figure 3.2). Both findings are in line with those of Hutter et al.[40] and Sobenin et al.[41] and can be explained by the fact that our artery samples contained end-stage lesions with no acute vascular event prior to the surgical intervention. In agreement, CASP3 was mostly seen in stable plaque areas, mainly as the uncleaved 32kd proenzyme, in a study by Kolodgie et al.[42]

When considering the activated 17kd fragment of caspase-3 (immunostaining for aCASP), however, we found strong expression in foam cells and macrophages in CKD lesions. This is in agreement with the observed higher inflammatory activity in these lesions as compared to those of the nonCKD group. Since CASP3 is linked to atherosclerotic lesion progression, its positive association with HO-1 comes as no surprise.

Next to hsCRP, lipid and hemoglobin levels, the biochemical characterization of the study subjects was complemented with an evaluation of the uremic retention solutes urea, creatinine, p-cresyl sulfate, indoxyl sulfate, PTH, phosphate and vitamin D. Although the levels of these variables were significantly different between CKD and nonCKD patients, none of them was associated with the atherosclerotic plaque characteristics. Also, F2-isoprostane levels, as stable markers of lipid peroxidation, were not different between CKD and nonCKD. This seems counterintuitive given the high inflammation and the presumed high oxidative stress in CKD as compared to nonCKD. However, it should be noted that all patients included in this analysis had clinically overt vascular disease, which may have obscured subtle oxidative stress differences related to kidney function. Moreover, circulating F2-isoprostane levels may not be representative of the local tissue oxidant/anti-oxidant ratio.

As an obvious consequence of the inclusion of patients scheduled for vascular surgery, our data are only applicable to advanced and clinically apparent atherosclerotic lesions, most probably at their morphological endpoint. Whether differences between CKD and nonCKD in morphology, immunohistochemistry and mRNA expression exists in earlier stages of atherosclerosis needs to be further addressed. Second, since this is a cross-sectional study it is not possible to infer cause and effect from our findings. Third, despite the successful inclusion of a large number of patients in the study protocol, the number of biopsies of sufficient quality for interpretation was rather limited. From this, we acknowledge that our study most probably lacks statistical power. However, we feel that our study points in a clear direction and that the power issue most probably results in underestimation rather than overestimation of the findings. Finally, the use of a semiquantitative scoring system for the immunohistochemical stainings and the complexity of the lesions do contribute to the power issue mentioned above. Nevertheless, the reliance on *in vivo* material from well-characterized patient groups, along with the availability of reliable, high quality extracted mRNA should be regarded as strengths of our study.

In conclusion, atherosclerotic lesions in CKD patients are characterized by higher inflammatory cell infiltration, more neovascularization and a higher proportion of plaque complications than those of nonCKD patients. CKD lesions had a tendency to lower expression of the anti-atherogenic enzyme HO-1. Further investigation of the potential effects of CKD on the expression and function of HO-1 and other anti-atherogenic enzymes is warranted.

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Online Supplement 3.1

Detailed materials and methods

Patients

One hundred and fifty-five patients (122 males, median age 69 [63-73]) who were hospitalized between February 2011 and September 2013 for elective open vascular surgery of the lower limb arteries or abdominal aortic aneurysm at the Department of Vascular Surgery of the University Hospitals Leuven, Belgium, provided informed consent to retrieve an arterial biopsy during the procedure. The patients were classified as nonCKD patients (n=109) (defined as eGFR (CKD-EPI formula) >60 ml/kg/1.73m² and exclusion of structural renal damage or proteinuria) and CKD patients (n=44; 10 category G3a, 14 category G3b, 5 category G4 , 15 category G5 (of whom 10 in dialysis) according to KDIGO classification.[16] Two patients with KDIGO CKD category G2 were excluded from further analysis. No other in- or exclusion criteria were used.
The study adhered to the principles of the Declaration of Helsinki and was approved by the ethics committee of the University Hospitals Leuven, Belgium.

Demographical, clinical and biochemical data

Demographical and clinical data were retrieved from the patient medical files through thorough file review by KD. For patients with CKD, primary renal diagnosis was defined according to the new ERA-EDTA Primary Renal Diagnosis (PRD) coding system.[17] Comorbidity was assessed using the method described by Charlson et al..[18] Moreover, the cardiovascular risk profile was calculated based on the presence or absence of Framingham Risk factors diabetes, AHT, smoking (current or quit <5years), obesity, dyslipidemia or positive familiar history of CVD, with a maximum score of 6.
Arterial hypertension was defined as a positive history reported in the medical file and/or current use of anti-hypertensive drugs. Diabetes was defined as a positive history reported in the medical file and/or current use of glucose lowering treatment. Dyslipidemia was defined as the presence of lipid abnormalities at admission (LDL cholesterol >115 mg/dL or high density lipoprotein (HDL) <40 mg/d or triglycerides >150 mg/dL) or current use of lipid lowering medication. Smoking status as well as the use of lipid lowering and anti-hypertensive treatment were derived from the admission file at day of surgery.
Cardiovascular disease status was defined based on the results of doppler/ultrasonography of the carotid and lower limb arteries (≥50% stenosis), magnetic resonance or computed tomography-based angiography of the lower limbs (≥ 2 arteries with moderate stenosis), electrocardiography, coronary angiography (≥1-vessel disease) , cyclo-ergometry, echocardiography and/or documented history of

acute myocardial infarction, stable or unstable coronary artery disease, peripheral artery disease, carotid disease with/without intervention. CVD grade was scored from 0-2 according to the number of diseased vascular beds (carotid disease, peripheral artery disease and/or coronary artery disease; '0': none; '1': one type of CVD; '2' 2 or more types of CVD).

Blood sampling and biochemical data

Blood samples were taken at the day of hospitalization or the day of surgery, before the surgical intervention.

Hemoglobin (g/dL), C-reactive protein (CRP) (mg/L), creatinine (mg/dL), urea (mg/dL), calcium (mg/dL), albumin (g/L), phosphate (mg/dL), bicarbonate (mmol/L), total cholesterol (mg/dL), HDL (mg/dL), LDL (mg/dL), triglycerides (mg/dL), 25-OH-vitamin D (µg/L)) were measured using standard laboratory techniques. Serum concentrations of full-length (biointact) PTH were determined by an immunoradiometric assay, as described elsewhere [19].
For further analysis on plasma and serum, blood was collected in vacutainer EDTA tubes and SST5 tubes (BD Diagnostics, Plymouth, UK), respectively.

F2-isoprostanes

Blood samples collected in EDTA tubes were kept at 4°C until centrifugation at 1860 g for 10 min at 4°C. The anti-oxidant BHT (butylated hydroxytoluene) was added to the plasma with a final concentration of 0.005%. All samples were treated within one hour after sampling and stored at -80°C until analysis. The total (free + esterified) concentration of the isoprostane 8-isoPGF2α (or iPF2αIII) was measured according to the methodology developed in the Laboratory of Nephrology, Department of Immunology and Microbiology, KU Leuven (HDL, unpublished).
Samples and calibration standards were prepared by an alkaline hydrolysis reaction before a solid phase extraction (SPE) cleanup procedure (Oasis MAX 96-well 30µm 60mg, Waters, Zellik, Belgium). The SPE procedure was carried out according to the manufacturer's instructions. The UPLC system was an Acquity H-class (Waters, Zellik, Belgium). Chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 x 2.1 mm; 1.7 µm particle size; Waters). Separation was performed isocratic with 55% (0.01% formic acid in milliQ water), 20% (methanol) and 25% (acetonitrile) at a flow rate of 0.6 ml/min, the total runtime was 12 minutes. Detection was performed using a Xevo TQS tandem mass spectrometer controlled with MassLynx software (Waters, Zellik, Belgium). Before ionization a post-column infusion of 5% ammonium hydroxide solution was combined with the column flow with a flow rate of 20µl/min. Ionization was achieved using electrospray in the negative ionization mode (ESI-) and the mass spectrometer was operated in multiple-reaction monitoring mode (MRM). The MRM transitions were 353.10→193.10 and 357.28→197.15 for

respectively iPF2αIII 8-isoPGF2α and the internal standard iPF2αII-d4 (or 8-isoPGF2α-d4. The lower limit of quantification was 0.03 ng/ml. The total, within-run, between-run and between-day imprecisions for 5 days were all < 10.5% for a low concentration and < 8% for a middle and high concentration. The mean recovery was 104%.

Uremic Toxins: p-cresyl sulfate, indoxyl sulfate

Upon arrival at the laboratory, SST5 tubes were centrifuged (1860xg) at 24°C for 10 min, after which serum was aliquotted and stored at –80°C until analysis. p-cresyl sulfate and indoxyl sulfate were quantified as described previously,[20] using a high-performance liquid chromatography system (Alliance 2695, Waters, Belgium), coupled to a Waters 2475 fluorescence detector. Limits of quantification of total serum concentrations were 2.39 µM for indoxyl sulfate and 4.41 µM for p-cresyl sulfate.

High-sensitive CRP (hsCRP) and interleukin 6 (IL-6) were measured in serum using enzyme-linked immunosorbent assays (hsCRP ELISA Demeditec, Kiel-Wellsee (Germany) and IL-6 HS ELISA e-Bioscience, Vienna (Austria)), performed according to the manufacturer's instructions.

Artery sampling, histologic analyses, RNA expression analysis

Arterial biopsies procured during vascular surgery were immediately divided in three parts: one part for paraffin sections, one part for frozen sections and the third part for storage on RNAlater stabilization fluid.

Histology and immunohistological analysis by light microscopy

For histologic examination by light microscopy (LM), formalin fixed paraffin embedded (FFPE) sections, 4µ thick, were prepared in a standard way. They were stained with hematoxylin and eosin (H&E). Immunohistochemical (IHC) stains included α-smooth muscle actin (αSMA), (mouse monoclonal, Ready-to-use, Dako), cluster of differentiation 68 (CD68) (anti-human CD68/KP-1, mouse monoclonal, Ready-to-use, Dako), HO-1 (Rabbit, 1/100, ENZO Life Sciences), MMP2 (mouse, 1/20, Oncogene), CD36 (Rabbit polyclonal, 1/50, Abcam), ICAM1 (Rabbit polyclonal, 1/20, Santa Cruz), VCAM1 (Mouse, 1/20, Dako), MCP1 (mouse monoclonal, 1/50, Abcam) and aCASP3 (Rabbit, 1/100, Abcam).

Histologic analysis by LM included 1. Identification of the type of atherosclerotic Lesions, 2. Identification of the type of complication and 3. Estimation of the evolution of the development of the former two. Lesions were defined and characterized according to the adapted AHA classification (Virmani et al.[21,22]). They are illustrated in Figures 3.1A-K. Basic, atherosclerotic lesions were classified as normal, intimal thickening (not shown), intimal xanthoma (IX, Figure 3.1A), pathological intimal thickening (not shown), fibrous cap atheroma (FCA, Figure 3.1B), Thin fibrous cap atheroma (TFCA, Figure 3.1Ci-ii) and fibrocalcific plaque (FCP, Figure 3.1D). Complications included plaque rupture (Figure 3.1E), erosion (Figure 3.1F), hemorrhage

(Figure 3.1G) and total occlusion (Figure 3.1H). A calcified nodule was encountered only once (not shown). Finally, evolution and maturity were classified based on H&E and αSMA. Evolution was classified in completely organized (Figure 3.1I) and actively remodeling lesions (Figure 3.1J-K). In the initial scoring matrix for maturity, lesions were judged as overall young, intermediate, old or a morphologic range. In the final analysis, lesions were classified as organized or still actively remodeling. Next, presence, location (media or (neo)intimal) and characteristics of plaque calcification was judged (spotty or nodular calcification) on the H&E sections. Fibrous cap characteristics, cap thickness and integrity of the cap, were judged on H&E and αSMA. Neovascularization was judged on H&E and αSMA by a semiquantitative scoring (0-3 with 0 'no neovascularization' to 3 'strong neovascularization). A selection of 26 sections was additionally stained for CD31 (Figure 3.4A-B). Presence of inflammatory cells was judged on the H&E staining (lymphocytes and eosinophils) and CD68 staining for mononuclear cells (macrophages and/or foam cells, score from 0-3, figure 4C-D). The degree of total inflammation was calculated by the sum of scores for macrophages, foam cells and lymphocytes (0-9). Presence of iron deposition was noted (0-1). Immunohistochemistry for HO-1, MMP2, aCASP3, CD36, MCP1, ICAM1 and VCAM1 was scored in a semiquantitative cell based manner by scoring from 0 (absent) to 3 (strong staining) in each cell type. Overall lesion staining intensity was then calculated from the sum of scores in the different cell types. As not all cell types had a positive score for each IHC staining, recalculation occurred with a final score from 0–3 using cutoff values 0:0, 1[1;x/3], 2[>x/3;2x/3] and 3[>2x/3;x] with x being the maximum score for the sum of scores for each IHC staining specifically.
Histologic analysis and scoring of the lesions was performed blindly by 2 independent experienced observers (KD, EV). A random sample of 20 biopsies was scored separately by each of them and scoring criteria were reproducible with high congruence (data not shown).

RNA extraction and gene expression analysis

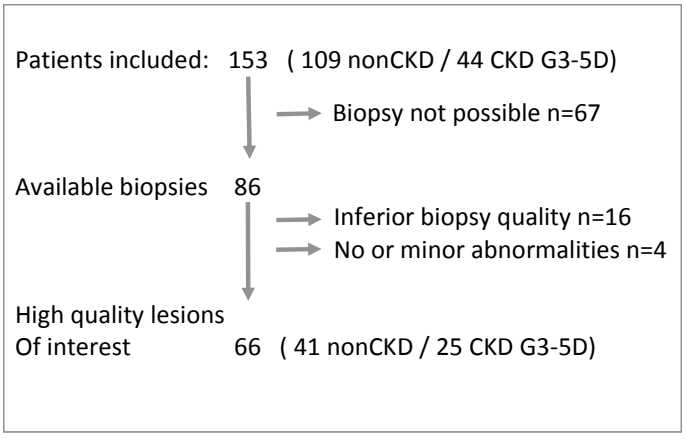
Part of the artery biopsies was transferred into RNAlater stabilization fluid and stored for 24 hours at 4°C, followed by storage at -20°C until extraction. Total RNA was extracted using the Rneasy Fibrous Tissue Mini Kit after tissue homogenization by the tissuelyser (7 mm beads) (Qiagen Benelux, Venlo, Netherlands). Resulting RNA-concentrations and purity of the RNA were determined by the use of the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). Integrity of the extracted RNA was verified using the BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) on a random subset of 20 arteries. The mean RIN value with the applied extraction method was 8±0.56. RNA samples were stored at -80° until Reverse Transcription to cDNA samples. For the generation of cDNA 50 ng of RNA was prepared with the SuperScript® VILO™ Master Mix in a total volume of 35 µL and ran on the Veriti PCR (Life techonologies, Gent, Belgium). Gene expression analysis was performed using

2 µL of the resulting cDNA and a Taqman Assay (7500 Fast Real-Time PCR System (Life techonologies) for HO-1 (Hs01110250), CD36 (Hs01567185), MCP1 (Hs00234140), ICAM1 (Hs00164932), VCAM1 (Hs01003372), MMP2 (Hs01548727) and CASP3 (Hs00234387) as Target genes and GAPDH as endogenous control gene.
Real-time measurements were recorded during 40 amplification cycles, and data were obtained as threshold cycle $C(_T)$ values. $C(_T)$ levels were <40 for all genes. Data were corrected for sample to sample variation in RNA reverse transcription reaction efficiency by normalizing the target mRNA quantity to GAPDH mRNA quantities. The relative expression level of the gene of interest was calculated using the $2^{-\Delta\Delta}C(_T)$ method and expressed as a Relative Quantitation (RQ=times upregulated) compared to a non-atherosclerotic nonCKD artery sample.

Statistics

Continuous variables are expressed as median and interquartile range. Categorical variables are expressed as percentages.
Comparisons between groups were made using non-parametric ANOVA (Wilcoxon Two-Sample test) for continuous data and Fisher's Exact test and Cochrane-Mantel Haenszel scores of association for categorical data. P-values <0.05 were considered significant.
For the multivariate analyses, missing values for PTH (n=7), serum phosphate (n=3) and albumin (n=3) were replaced by their respective mean values from either the nonCKD or CKD group. For the CKDG5D patients, eGFR was arbitrarily set on 5 ml/min/1.73m² (n=6).
Based on the findings of the comparative analysis, logistic regression analysis was performed to define variables associated with plaque complications. Univariately associated variables on P<0.2, variables that differed on P<0.2 between nonCKD and CKD and the established cardiovascular risk factors sex, age, diabetes, BMI, AHT, smoking status and dyslipidemia were introduced in a multivariate analysis. The stepwise technique was used to identify the best subset on P<0.2. This subset was then subjected to a final backward selection elimination procedure on P<0.05.
Furthermore, determinants of neovascularization and HO-1 expression were explored in detail using univariate Spearman Rank correlation statistics, followed by multivariate linear regression analysis. The multivariate analysis included all variables found to be univariately associated with the outcome variable on P<0.2, variables that differed on P<0.2 between nonCKD and CKD and established cardiovascular risk factors, regardless of their association with the outcome variable: sex, age, diabetes, AHT, smoking status, dyslipidemia and BMI. After excluding collinearity, the best subset of variables was selected by stepwise selection on P<0.2. This subset was then subjected to a final backward selection elimination procedure on P<0.05. Inspection of residual plots assured that the a priori assumptions for linear regression were justified.
The SAS (version 9.3, the SAS institute, Cary, N.C., USA) software package was used for the statistical analyses.

Online Supplement 3.2



Flow chart patient inclusion and biopsy retrieval

Online Supplement 3.3

Results: General pathology

Immunohistochemistry for HO-1, MMP2, aCASP3, MCP1, CD36, VCAM1 and ICAM1

Immunostaining shows strongest HO-1 expression in foam cells and macrophages, followed by endothelial cells and fibroblasts. The weakest HO-1 staining is seen in the vascular smooth muscle cells (VSMC). Staining for MMP2 reveals positivity in all cell types except VSMC, with the strongest signal in foam cells and macrophages followed by fibroblasts and endothelial cells. Activated caspase-3 (the 17kd-fragment) is only positive in the mononuclear cells, with strongest expression in foam cells located at organized areas of remodeling plaques. MCP1 staining is predominantly positive in mononuclear cells (most in foam cells). CD36 is mainly positive in mononuclear cells and to a lesser degree in endothelial cells and fibroblasts. CD36 colocalizes with aCASP3 and MCP-1. Positive VCAM1 staining is only seen in endothelial cells, and exceptionally in foam cells. ICAM1 is expressed in all cell types except VSMC, with the strongest signal in the endothelial cells.

Gene expression analysis of HO-1, MMP2, CASP3, MCP1, CD36, VCAM1 and ICAM1

All 66 samples from nonCKD and CKD patients are referenced against a nonCKD nonatherosclerotic reference sample. The RQ values are categorized as follows: RQ<0.5: significant downregulation, 0.5<RQ<2.5: equal gene expression, 2.5<RQ<50: mild upregulation and RQ>50: strong upregulation of the target gene from the atherosclerotic as compared to the nonatherosclerotic control. Overall, HO-1, MMP2, CD36 and ICAM1 genes are significantly upregulated as compared to the non-atherosclerotic control (median RQ resp. 6.5 [3.3-21.3]; 72.6 [45-130]; 37 [18-58] and 37 [28-52]). Caspase-3, MCP1 and VCAM1 gene expression is not different between atherosclerotic samples and the non-atherosclerotic sample (median RQ resp 0.74 [0.55-0.92]; 0.89 [0.41-1.72] and 2.01 [1.3-2.9]).

CHAPTER 4

Heme oxygenase-1 overexpression mitigates oxidative stress-induced apoptosis in uremic endothelial cells

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Submitted

Abstract

Increased oxidative stress is postulated to be the unifying concept in chronic kidney disease (CKD) related inflammation, endothelial dysfunction and atherosclerosis. Heme oxygenase-1 (HO-1) has important antioxidant and anti-inflammatory properties, attenuating atheroma formation. We studied the toxic response and HO-1 expression of primary human umbilical arterial endothelial cells (HUAECs) exposed to oxidative stress, preconditioned with healthy or uremic human serum. The impact of HO-1 overexpression on oxidative stress-dependent outcomes was analyzed. HUAECs were preconditioned for 72 hours with healthy (HS) or uremic human serum (US). Cells were exposed to Hemin as a HO-1 inducer and to peroxynitrite or SIN-1 as sources of oxidative stress. HO-1 mRNA and protein expression were analyzed as were viability and apoptosis, using MTT, western blots for caspase-3 and TUNEL staining. Reactive oxygen species (ROS) formation was determined by Cellrox and Mitosox. Endothelial cell activation was evaluated by vascular cell and intercellular adhesion molecule-1 and matrix metalloproteinase-2 mRNA expression. HO-1 expression is not differentially affected in HS as compared to US, when induced by Hemin, $ONOO^-$ or SIN-1. SIN-1 induces cytoplasmic ROS to a comparable degree in HS and US, but induces higher cell apoptosis in US. HO-1 overexpression quenches SIN-1 induced cROS formation and abrogates the higher vulnerability imposed by uremia. We conclude that uremic preconditioning predisposes for apoptosis during exposure to oxidative stress. Hemin-induced HO-1 overexpression mitigates uremia-induced cellular vulnerability and decreases cROS generation. This finding inspires further investigations to explore the therapeutic potential of agents that activate HO-1 to mitigate uremic vascular injury.

Introduction

Increased oxidative stress has been postulated to be the unifying concept of chronic inflammation, endothelial cell activation and endothelial dysfunction in chronic kidney disease (CKD) related accelerated atherosclerosis.[1] Endothelial dysfunction associated formation of peroxynitrite ($ONOO^-$) plays an important role in the initiation of atherogenesis and protein nitration, "the footprint of $ONOO^-$ generation", has been found in atherosclerotic lesions.[2,3]

Oxidative stress results from an imbalance between oxidants and antioxidants, in favor of the oxidants, in an otherwise well-controlled balance between both.[4,5] Indeed, reactive oxygen/nitrogen species (ROS/RNS) at moderate concentrations act as signaling molecules in cell metabolism and survival pathways. For example, SIN-1 or 3-morpholinosydnonimine, an endogenous $ONOO^-$ donor, has been shown to be non-toxic and even cytoprotective in a narrow concentration range up to 5 mM, but cytotoxic and lethal in 100% of the cells at 10 mM when present for 12 hours.[6] Intermediate concentrations cause variable cytoxicity after longlasting exposure, indicative of a delicate balance between ROS/RNS-mediated cell signaling and cumulative damage in the vascular bed.[7]

Against this background, the study of antioxidants in the treatment and prophylaxis of atherosclerosis in CKD is an attractive area of clinical research.[8-10] Unfortunately, several clinical trials studying antioxidant supplementation in CKD failed to show improved outcome, suggesting insufficient potency in protecting against cardiovascular disease.[11]

The enzymatic antioxidative arsenal of the human body in CKD-related atherosclerosis has been incompletely studied[12,13] and remains therefore relatively unexplored. Exposure of cells to ROS/RNS such as $ONOO^-$ leads to activation of *nuclear factor erythroid 2-related factor 2* (*NRF2*), with subsequent induction of genes encoding protective enzymes such as *NAD(P)H quinone oxidoreductase 1* (*NQO1*) or *Heme oxygenase-1* (HO-1).[14] HO-1 is responsible for the rate-limiting enzymatic degradation of heme to free ferrous iron, carbon monoxide (CO) and biliverdin, the latter being rapidly converted by biliverdin reductase to bilirubin. Each of these enzymatic end-products exerts antioxidative, anti-inflammatory and anti-apoptotic effects through different mechanisms and, amongst other favorable outcomes, is able to attenuate atheroma formation.[15-17] Hemin, a registered orphan drug for the treatment of attacks of acute intermittent porphyria, is a strong and selective inducer of *HO-1*.[18] Circulating serum concentrations under treatment reach up to 100 μM.

Limited evidence from remnant kidney models shows decreased *NRF2* (and consequently *HO-1*) activity despite increased oxidative stress and inflammation in the

context of CKD.[19] As this model focused on kidney tubular cells, however, it does not provide information regarding NRF2 and HO-1 in the vascular bed.
The aim of the present study was to investigate how uremia affects oxidative stress-induced cell toxicity and expression of HO-1 in primary endothelial cells. For this purpose, human umbilical arterial endothelial cells (HUAECs) were grown in uremic (US) and healthy serum (HS) and subsequently exposed to $ONOO^-$ or SIN-1 to simulate initial exposure to oxidative events of atherogenesis in uremia. As a secondary aim of our study, the impact of Hemin-induced HO-1 overexpression was investigated in uremia on oxidative stress-mediated endothelial cell toxicity and apoptosis .

Concise Methods

For reagents, materials and detailed methodologic protocols we refer to the online supplement 'detailed materials and methods'.

Serum pools

For the preconditioning of the HUAECs (Promocell, Bio-Connect, Huissen, Netherlands), serum was pooled from healthy volunteers (HS) (n=10) or from hemodialysis patients (US) (n=40). Blood sampling in dialysis patients was performed immediately prior to dialysis. Biochemical characteristics of the serum pools are given in Table 4.1.

Table 4.1 Biochemical characteristics of the serum pools.

	Heatlhy serum	Uremic serum
Creatinine, mg/dL	0.85	7.68
Ureum, mg/dL	28.5	119.5
Albumin, g/L	48.7	39.4
CRP, mg/L	1.05	16.90
Glucose, mg/dL	83	109
Ferrous iron, µg/dL	93	48
Ferritine, µg/L	106	301
LDH, U/L	195	233
Free Hemoglobin, mg/dL	5	5
Cholesterol, mg/dL	181	182
HDL, mg/dL	57	38
Triglycerides, mg/dL	102	127

Summary variables are presented. CRP: C-reactive protein, LDH: Lactate Dehydrogenase, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein.

Cell culture conditions

All HUAEC cultures were performed in gelatin-coated flasks or in 6-96 well-plates (Cellstar, Greiner Bio-One, Vilvoorde, Belgium). HUAECs were grown in T75 flasks till 90% confluence in a 5% CO_2 incubator at 37°C. Cells were then seeded at 50% confluence in 6-96 well plates and grown in basic EBM2-medium supplemented with 30% healthy (HS) or uremic serum (US) for 72 hours, until 90% confluence was reached. All following experiments were conducted on HS- or US-preconditioned cells.

Exposure of HUAEC to $ONOO^-$, SIN-1 and hemin

Cells were exposed to different concentrations of Hemin (25, 50 and 100 µM, dissolved in DMSO), a specific *HO-1* inducer and/or to $ONOO^-$ as a transient oxidative stressor (concentration range 0.1 to 1 mM) or SIN-1 as a source of chronic oxidative stress (7.5 and 10 mM dissolved in PBS). During its decomposition, this water-soluble metabolite of molsidomine releases $O_2^{\cdot -}$ and $NO^{\cdot}$, which spontaneously recombine to form the highly reactive molecule $ONOO^-$. The use of SIN-1 allows for a more gradual and continuous release of $ONOO^-$ and longer exposure than achievable with $ONOO^-$, which decomposes rapidly.[20] At low concentrations, SIN-1 exerts cytoprotective effects within a narrow range (1-5 mM).[6] However, starting from 7.5 mM, SIN-1 was shown to be cytotoxic, leading to excessive $ONOO^-$ formation and cell toxicity, including induction of apoptosis.[6] Therefore, we compared SIN-1 at 7.5 and 10 mM to simulate increasing oxidative stress, encountered during early atherogenesis, including oxidative stress-mediated cell responses and cell toxicity.

To assess the effect of Hemin-induced HO-1 expression on the SIN-1 induced oxidative stress response, cells were first incubated for 5 hours with 50 µM Hemin, followed by exposure to SIN-1 (7.5 or 10 mM) for the mentioned time. Blanks and controls were analyzed with and without the addition of DMSO to the medium.

RNA extraction and qRT-PCR

After 6 hours of exposure to SIN-1 ± Hemin, RNA was extracted from trypsinized cell pellets and reverse transcribed. The resulting cDNA was diluted 10-fold. For *HO-1*, each PCR was carried out in duplicate on a 7500 Fast Real-Time PCR system (Applied biosystems, Gent, Belgium). Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was determined as a stable endogenous control for each sample. One healthy serum sample was selected as an internal reference for every gene expression analysis. The relative gene expression was calculated by comparing cycle times for target PCR using the following equation: relative gene expression = $2^{-(\Delta CTsample-\Delta CTcontrol)}$.[21]

Reverse transcribed (RT)-PCR products and primer sequences for *HMOX1* and *GAPDH*, purchased from IDT (Leuven, Belgium) are shown in Table 4.2. A Taqman Assay (7500 Fast Real-Time PCR System (Life technologies, Gent, Belgium) was used for *MMP2, VCAM1 and ICAM1*. *GAPDH* was used as endogenous control gene.

Table 4.2 HO-1 and GAPDH primers.

Target gene	Forward sequence	Reverse sequence
HO-1	5' AACTTTCAGAAGGGCCAGGT 3'	5' CTGGTGTGTAGGGGATGACC 3'
GAPDH	5' TGGTATCGTGGAAGGACTCATGAC 3'	5' ATGCCAGTGAGCTTCCCGTTCAGC 3'

Protein extraction and western blot analysis

After 8 hours of exposure to SIN-1 ± Hemin, cells of 3 wells were scraped in 150 µL lysis buffer. The total protein concentration was determined by Bradford quantification.[22] Proteins were transferred to nitrocellulose. Membranes were incubated with various primary antibodies: anti-HO-1 (rabbit pAb, Enzo life sciences ALX-210-116 1/1000), anti-β-Actin (rabbit mAb, Cell signaling technology 4970S 1/1000) and anti-caspase-3 (Rabbit mAb, Cell signaling technologies 9665 1/1000). After adding horseradish peroxidase–conjugated secondary antibodies (Goat anti-Rabbit HRP 1/1000), immunoreactive bands were visualized by ECL and detected on a chemiDoxTM XRS+ Molecular Imager with Imager LABTM software (Bio-rad, Temse, Belgium) .

Cell viability and apoptosis

MTT assay

Endothelial cells were exposed to $ONOO^-$ in increasing concentrations from 0.1 to 1 mM. After 6 hours, cells were analyzed for viability by the MTT assay, a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble formazan, giving a purple color. MTT was added at 1 mg/mL to the treated cells. After incubation for 2-3 hours, when color conversion occurred, MTT solubilization solution was added and 30 minutes later, colorimetric conversion was read at 550-630 nm on an EL808 Spectrophotometer (Bio-tek, Bad Friedrichshall, Germany).

TUNEL staining

Cells were exposed to SIN-1 ± Hemin. Endothelial cell apoptosis was estimated by a Cell Death Detection Kit (Roche Applied Science, Brussels, Belgium), according to the manufacturer's instructions, using Flow cytometry analysis (Facs Canto II, BD Biosciences, Erembodegem, Belgium). Mean Fluorescense intensities were calculated.

Values were normalized to an internal control Healthy sample. The relative fold increase of the mean Fluorescence intensity is shown in the figures.

Measurement of ROS: Cellrox® and Mitosox®

To detect mitochondrial (mROS) and cytoplasmic ROS (cROS) in treated HUAECs, measurements of MitoSOX Red® and CellROX Deep Red® (Invitrogen, Life Technologies, Gent, Belgium) fluorescence intensity in a Flexstation® 3 Microplate reader (Molecular Devices, Berkshire, United Kingdom) were performed. After treatment with Hemin ± SIN-1, cells were incubated with PBS containing 5 µM MitoSOX® or CellROX®. Labeled cells were washed and analyzed according to the manufacturer's instructions.

Statistics

For all experiments, comparisons were performed by nonparametric ANOVA (Wilcoxon Two-Sample test). Differences were considered significant at $P<0.05$.

Results

Induction of HO-1: mRNA and protein expression analysis

Preconditioning of HUAECs with HS or US for 72 hours does not affect *HO-1* mRNA expression. Peroxynitrite, SIN-1 and Hemin all cause a marked increase in *HO-1* mRNA (Figure 4.1A, C, D), in a dose- and time-dependent manner (Figure 4.1B). *HO-1* upregulation is maximal at 6 hours and normalizes to baseline after 24 hours (data not shown). Peroxynitrite up to 1 mM induces *HO-1* mRNA up to 45-fold (HS/US vs. control, $P=0.010/0.013$ respectively, Figure 4.1A). Because $ONOO^-$ is highly reactive, with a very short half-life,[23] a more stable 'chronic' low-grade exposure to oxidative stress was applied by the $ONOO^-$ donor SIN-1. SIN-1 at 7.5 and 10 mM induces *HO-1* mRNA up to 88-fold (US and HS vs. control $P<0.0001$, Figure 4.1C), although less pronounced at 10 mM (up to 52-fold), probably related to the higher occurrence of apoptosis at this concentration (see Figure 4.1C and 4.2B). Hemin 50 and 100 µM potently induce *HO-1* mRNA up to 196-fold (HS vs. control $P=0.0007$, Figure 4.1D). These findings are confirmed by western blot analysis (Figure 4.1E). In agreement with the strongest *HO-1* mRNA induction by 100 µM Hemin (US and HS Hemin vs. 7.5 mM SIN-1 ($P<0.0001$)), western blots confirm the highest HO-1 expression after the combined treatment with Hemin preceding 7.5 mM SIN-1.

For none of these HO-1 inducing agents a difference in HO-1 mRNA or protein expression between US and HS is found: uremia per se does not influence oxidative stress-mediated HO-1 expression.

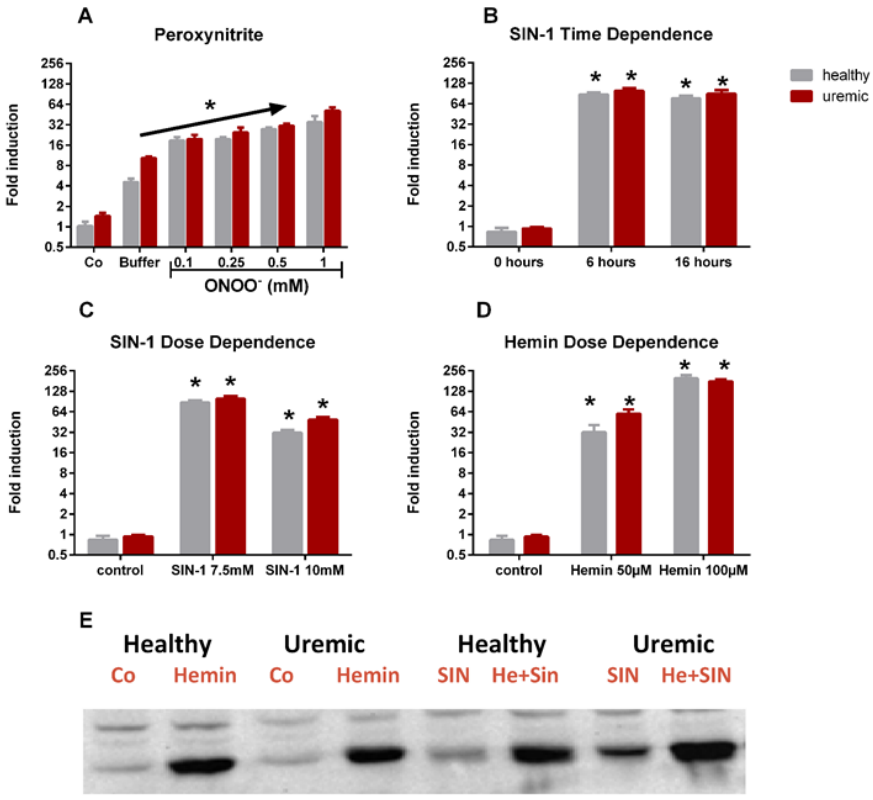

Figure 4.1 Induction of HO-1 expression by $ONOO^-$, SIN-1 and Hemin in HUAECs. (A) Fold-upregulation of HO-1 mRNA in HUAECs after 6 hours of incubation with the indicated concentrations of $ONOO^-$, compared to an internal healthy control HUAEC sample (n=3 in each condition. (B) Time-effect (6 and 16 hours) for the fold-upregulation of HO-1 mRNA expression by 7.5 mM SIN-1, (n=9 in all cases). (C) Concentration-effect for the fold-upregulation of HO-1 mRNA expression by 7.5 and 10 mM SIN-1 after 6 hours of incubation (n=9 in all cases) . (D)) Fold-upregulation of HO-1 mRNA in HUAECs after 6 hours of incubation with the indicated concentrations of Hemin (n=6 for Hemin 50 µM and n=10 for Hemin 100 µM). (E) Western blot for HO-1 in HUAECS after exposure to Hemin (Hemin or He: 100 µM), SIN-1 (SIN: 7.5 mM) or their combination (He+SIN) in HUAECs after 9 hours of incubation. In all cases HUAECs were preconditioned in EGM-2 MV medium containing 30% normal human serum (Healthy, HS) or uremic serum (Uremic, US) for 72 hours, as indicated. Mean values ± SEM are shown. (* $P \leq 0.01$ compared to healthy control).

Cell toxicity: viability, apoptosis and necrosis

Preconditioning with HS or US does not influence cell viability, as evaluated by MTT assay (Figure 4.2A). Uremia per se does not lead to increased cell death. Peroxynitrite causes a concentration-dependent cell toxicity, detected in the MTT assay. This cell toxicity is significantly higher in US- compared to the HS-preconditioned cells at $ONOO^-$ 0.1-0.5 mM (43% vs. 23%, $P<0.05$). At the highest concentration (1 mM) cell-toxicity levels off and is equal in both conditions. There is no influence of the NaOH buffer (solvent for $ONOO^-$) on the measured cell viability. To further investigate the nature of this cell toxicity, we incubated preconditioned HUAECs with SIN-1 at 7.5 and 10 mM and analyzed these cells 8 hours later for the occurrence of apoptosis, performing a TUNEL analysis via flow cytometry. US preconditioning predisposes HUAECs towards apoptosis to a greater extent than HS preconditioning. (Figure 4.2B) At 7.5 mM SIN-1, the mean fold increase (MFI) in TUNEL signal was 5.23 in US (US SIN-1 7.5 mM vs. HS control, $P=0.002$), whereas there is hardly any apoptosis in HS (MFI 1.74; HS SIN-1 7.5 mM vs. HS control $P=0.41$; HS vs. US at SIN-1 7.5 mM $P=0.036$). At 10 mM, SIN-1 is toxic in both conditions and strongly triggers apoptosis (MFI US 11.82 and HS 9.02; US vs. control $P=0.008$, HS vs. control $P=0.004$). Western blot analysis reveals formation of cleaved caspase-3 in US, confirming activation of apoptosis by SIN-1. Hemin does not negatively affect cell viability (not shown) and does not trigger the appearance of cleaved caspase-3 in western blots (Figure 4.2C). On the contrary, pre-incubation of HUAECs with 50 µM Hemin abrogates the SIN-1 induced apoptosis (Figure 4.2B) and restores cell viability in both groups (TUNEL US hemin+SIN-1 7.5 mM vs. US SIN 7.5mM $P=0.015$; US Hemin + SIN-1 10mM vs HS control $P=0.119$; US hemin+SIN-1 7.5 vs. control $P=1.00$). Likewise, Hemin pretreatment reduces appearance of the SIN-1-induced cleaved caspase-3 in western blots (Figure 4.2C) in uremia.

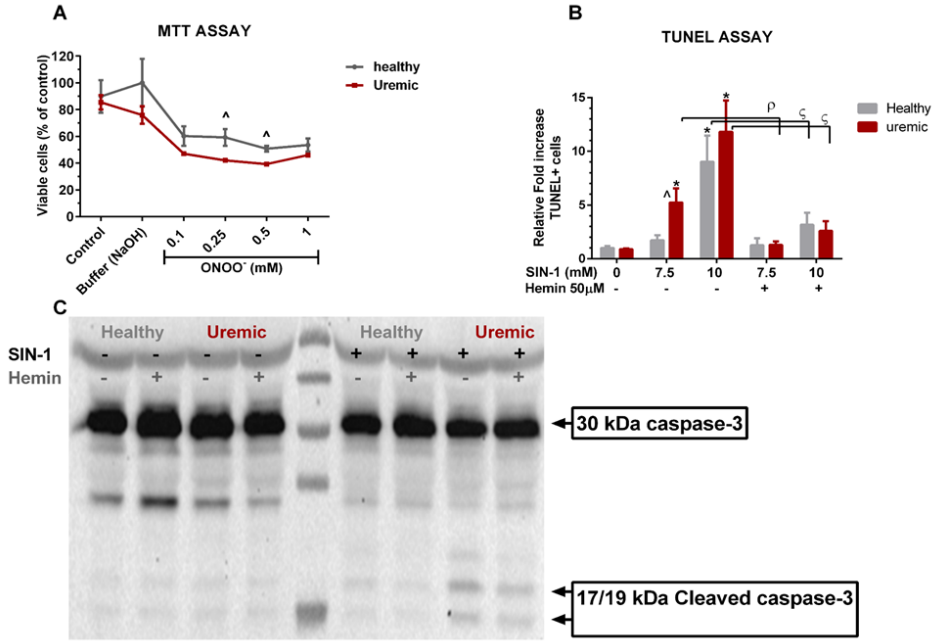

Figure 4.2 HUAEC survival after exposure to SIN-1: protection from apoptosis by Hemin. *(A) MTT assay of HS (healthy serum) or US (uremic serum) preconditioned HUAECs exposed to the indicated concentrations of $ONOO^-$. Cell viability is more impaired in US preconditioned HUAECs, at $[ONOO^-]$ = 0.25 and 0.5 mM (^). (B) (TUNEL staining after 9 hours of HUAEC incubation with SIN-1 at the indicated concentrations. Pretreatment with 50 µM Hemin for 5 hours largely abrogates the SIN-1 triggered apoptosis. Apoptosis is shown as the Mean Fluorescence Intensity ± SEM, expressed relative to the signal for an internal healthy control sample (n=5-6 in each condition). (C) Western blot analysis for intact and cleaved caspase-3 showing strongest induction of cleaved caspase-3 by SIN-1, largely abrogated by pretreatment with 50 µM Hemin for 5 hours in uremia preconditioned HUAECS, as indicated. (^ P<0.05 for comparison HS vs US preconditioning; *p<0.01 for comparison with healthy baseline control; ρ P< 0.05 vs. non-Hemin treated HUAEC cultures at 7.5 mM SIN-1; ς P< 0.01 vs. non-Hemin treated HUAEC cultures at 10 mM SIN-1).)*

ROS measurements: cROS and mROS

To better understand the mechanism rendering uremia preconditioned cells more prone to apoptosis induction by low-grade oxidative stress, both mitochondrial and cytoplasmic reactive oxygen species were measured by means of Mitosox (mROS) and Cellrox (cROS) in basal conditions and after exposure of HUAECs to SIN-1 and/or hemin. First, there are no differences in mROS or cROS generation after preconditioning for 72 hours with HS or US (HS vs US mROS *P*=0.25 and cROS *P*=1.00): uremia per se does not cause more cROS formation. At 50 µM, Hemin pretreatment decreases mROS

formation vs. control in US (*P*=0.007). There is a non-significant tendency to decrease in mROS vs. control in HS (*P*=0.068). Hemin 25 and 50μM do not impact cROS formation (Figure 4.3B; HS *P*=0.61, US *P*=0.15). The addition of 7.5 mM SIN-1 decreases mROS formation in both HS (*P*=0.006) and US (*P*<0.001) as compared to control. In contrast, with SIN-1 at 10 mM no reduction of mROS is observed in HS (*P*=0.28) and US (*P*=0.49). As expected, the addition of SIN-1 increases cROS formation vs. control at both 7.5 (US and HS *P*=0.0004) and 10 mM (US *P*=0.0004, HS *P*=0.0022). There are no significant differences in SIN-1 or Hemin induced mROS or cROS generation between HS and US. Hemin pretreatment followed by SIN-1 7.5 mM decreases mROS formation as compared to control, and this to the same extent as SIN-1 or Hemin alone (US *P*=0.004, HS *P*=0.0187). In addition, Hemin pretreatment prevents SIN-1-induced cROS formation (HS *P*=0.0001, US *P*=0.0004). In US conditions, this is however only a partial effect and cROS remain higher as compared to the control conditions (*P*=0.002).

RNA expression of adhesion molecules

RNA expression analyses of matrix metalloproteinase-2 (*MMP2)* vascular cell adhesion molecule-1 (*VCAM1)* and intercellular adhesion molecule-1 (*ICAM1)* were performed. There are no differences in the expression of MMP2, *VCAM1 or ICAM1* between HS and US after 72 hours of preconditioning (US vs. HS, *VCAM1* P=0.78, *ICAM1 P*=0.30, *MMP2 P*=0.31).

SIN-1 7.5 mM induces a non-significant 2-fold increase in *MMP2* mRNA (*P*=0.066 for both HS and US). Hemin induces *MMP2 mRNA* as compared to control (US *P*=0.04 and HS *P*=0.026), but Hemin pretreatment followed by 7.5mM SIN-1 reduces *MMP2* mRNA expression back to basal levels (Hemin+SIN-1 7.5 mM vs. Hemin: US *P*=0.0218 and HS *P*=0.0499) and strongly reduces *VCAM1* expression in both HS and US (*P*=0.0007 and 0.0004, respectively).

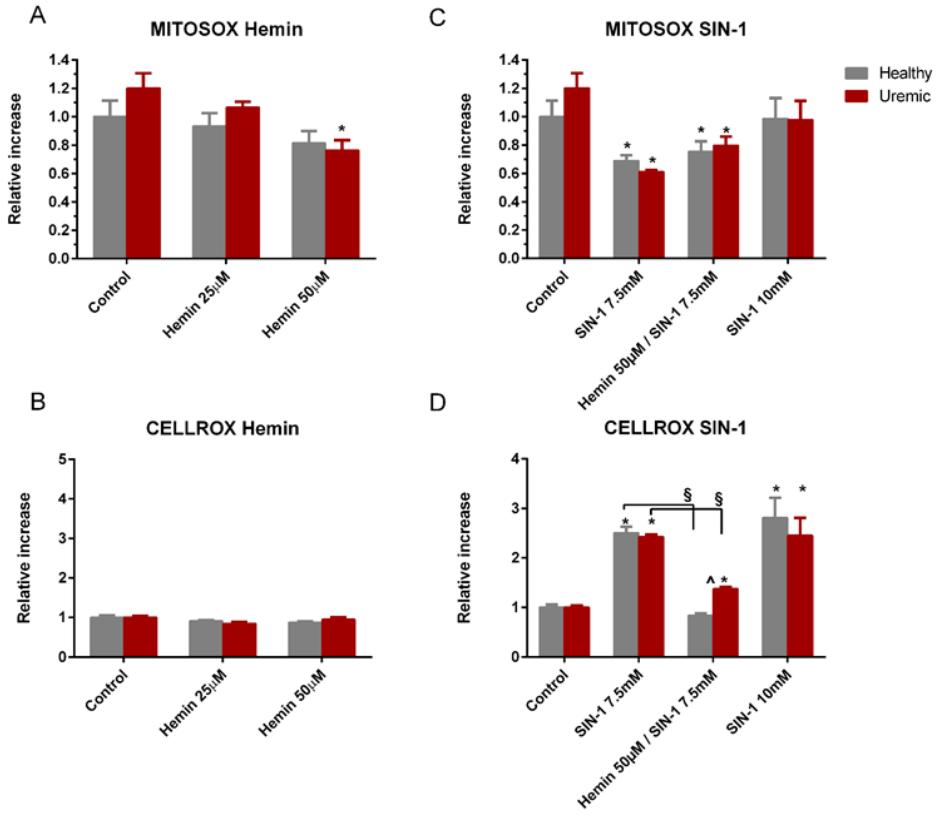

Figure 4.3 HUAEC mitochondrial and cytoplasmic oxidative stress by exposure to SIN-1. Mitosox (A, C) and Cellrox (B, D) detected fluorescence intensity in HUAECs after 4 hours of treatment with Hemin and/or SIN-1 at the indicated concentrations, expressed as a fold-induction of fluorescence intensity vs a healthy control (n=12 for control, 10 mM SIN-1 and 25 or 50 µM Hemin and n=18 for 7.5 mM SIN-1 and Hemin + SIN-1). (* P<0.02 vs. healthy control; ^ P<0.01 for HS vs. US preconditioning; § P<0.001 vs. non-Hemin treated HUAEC cultures at 7.5 mM SIN-1).

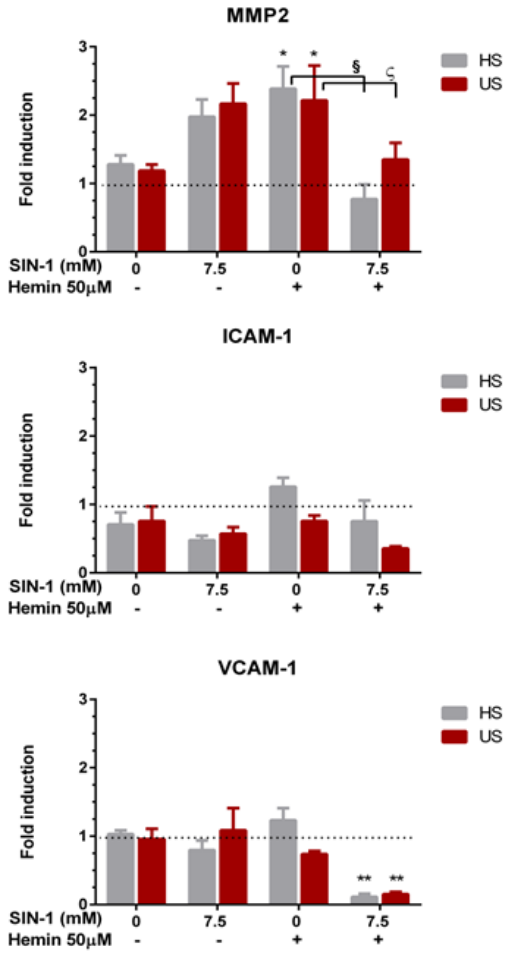

Figure 4.4 Transcription regulation by SIN-1 of pro-infammatory mediators in HUAECs. *Fold-induction of mRNA expression for* MMP2 *(A),* ICAM1 *(B) and (C)* VCAM1 *in HS- or US-preconditioned HUAECs, after exposure to 50 µM Hemin, 7.5 or 10 mM SIN-1 or both for 6 hours, as indicated. RNA expression analysis was performed using a Taqman assay for* GAPDH *and the respective genes, in duplex analysis vs. a healthy control, represented by the dotted line. (* P<0.05 and ** P<0.001 compared to healthy control condition; § P<0.05 for combined Hemin + SIN-1 vs. Hemin in healthy conditions ; ç P<0.01 for combined Hemin + SIN-1 vs. Hemin in uremic conditions).*

Discussion

The main finding of the present study is that arterial endothelial cells in uremia show a higher vulnerability to the oxidative stressor $ONOO^-$ as compared to endothelial cells exposed to healthy serum. Hemin-induced HO-1 overexpression mitigates this cell vulnerability in uremic conditions by normalizing apoptosis to baseline levels, linked to a reduction of cytoplasmic ROS generation.

HO-1 has a well-known role in protection against atherosclerosis, which is accelerated in CKD.[24,25] Impaired HO-1 expression was seen in the context of CKD in a remnant kidney mouse model.[19] Therefore, we aimed to investigate the impact of uremia on the oxidative stress-induced expression of HO-1 and on endothelial cell toxicity in an arterial endothelial cell model.

We found that HUAECs in uremic and healthy conditions manifest comparable expression of HO-1 and that its expression is triggered to the same extent in response to oxidative stress. Likewise, preconditioning of the HUAECs with either healthy or uremic serum does not result in differential mRNA expression of the adhesion molecules *MMP2*, *VCAM1* and *ICAM1*, important mediators of chronic endothelial inflammation. Moreover, no increase in ROS generation or apoptosis is found when endothelial cells are grown in otherwise unstressed uremic conditions.

In both uremic and healthy conditions, exposure to 7.5 mM SIN-1 induces cROS, but decreases mROS, and this does not result in endothelial cell activation, as judged from *VCAM1* and *ICAM1* mRNA expression levels. In contrast, 10 mM SIN-1 induces cROS with generalized cell death in both conditions, as described earlier.[6]

These findings are in line with those of Mattart et al who showed that SIN-1 in low doses is protective by a.o. ROS mediated activation of *NRF2* and ARE-driven genes such as *HO-1* and by the activation of a defensive autophagy mechanism.[6] Indeed, ROS in small amounts can be beneficial and function as messengers for the induction of signaling pathways[26] whereas in higher concentrations ROS become toxic and lead to oxidative damage and apoptosis.

Interestingly however, when exposed to increasing concentrations of the short-lived oxidative stressor $ONOO^-$ and to SIN-1 at 7.5 mM, cell toxicity and apoptosis occur at lower concentrations of the oxidants and to a larger extent in uremic conditions. In uremic conditions, 7.5 mM SIN-1 induces apoptosis through a caspase-3 mediated pathway. This implies that oxidative stress exerts more cellular stress on endothelial cells in uremic than in healthy conditions. However, the induction of HO-1 is not different when directly measured in these cells, and the direct measurements of cROS and mROS are comparable in HS and US-preconditioned cells.

The measured cytoplasmic ROS in our experiments is considered mainly the result of the release of $ONOO^-$ by the degradation of SIN-1. Next to this molecule, cROS can however be generated by a variety of cellular enzyme systems.[27] Mitochondrial ROS result from the mitochondrial respiration and electron leakage and are implicated in aging as well as in a range of degenerative diseases.[28] The effect of the generated cROS and changes in mROS on the generation of oxidation end-products such as nitrotyrosine, DNA damage or on the generation of non-radical oxidizing molecules participating a.o. in lipid oxidation, however, was not quantified in the present study.

Uremia has often been shown to lead to a pro-oxidative state. We find just a minor, nonsignificant increase of mROS after uremic preconditioning as compared to healthy conditions, but this is obviously just a small piece of the puzzle of the human oxidant/antioxidant mechanisms. A proteomics study by Carbó et al found indeed upregulated antioxidant genes such as superoxide dismutase (SOD) and gluthathione peroxidase (GPx) after the exposure of human umbilical venous endothelial cells (HUVECs) to uremic serum.[29] At the same time however, decreased SOD and GPx activities in uremia have been described.[12,30] Also, deficiency of ROS scavengers such as ascorbic acid, tocopherol, bilirubin and albumin have been described in CKD.[1,13] Others reported increased oxidative stress in cells exposed to uremic toxins.[31,32] Of course there are additional uremic features that contribute to an increased vulnerability to oxidative stress such as the inflammatory state of CKD. We find indeed higher CRP levels in the uremic serum pool. However, preconditioning with US, containing more (low-grade) inflammation than HS, did not result in a higher HO-1 expression in the uremic cells. This could be interpreted as a relative failure of antioxidative defense mechanisms in uremia, with subsequent oxidative damage.

Notwithstanding this interpretation, we found that an interventional strategy, consisting of strong induction of HO-1 was capable of abrogating most of the apoptotic manifestations, including the occurrence of cellular oxidative stress. These findings clearly establish a relation between cellular integrity and oxidative/antioxidative imbalance in uremia, an imbalance that can be restored by artificially raising the cytoplasmic HO-1 concentrations to high levels. As a result, excessive oxidative stress appeared to be abolished, apoptotic tendencies reduced and inflammatory adhesion molecules such as *VCAM1* be reduced, slowing down endothelial inflammation. Our findings uncover an interesting possibility for potential therapeutic interventions. Indeed, as Hemin is available as a registered drug for humans in acute intermittent porphyria, its use for potent HO-1 induction in other medical indications would broaden its field of applicability. In a recent phase IIb study Hemin preconditioning was evaluated in deceased donor renal transplantation for its potential to reduce ischemia-reperfusion related injury.[33] It remains to be further explored whether and by which

regimen such a treatment could be useful in a more chronic process like atherogenesis. Moreover, even when the protective effect of HO-1 overexpression in our model of arterial endothelial cells appears to be coupled to its antioxidant properties, further mechanistic studies are needed to confirm this conclusion.

Probably one of the most important limitations of this study is the lack of quantification of oxidation end products. $ONOO^-$ leads typically to the generation of nitrotyrosine.[20] Protein nitration is considered the 'footprint' of $ONOO^-$ generation but also lipids, DNA and enzymes could be oxidatively modified by $ONOO^-$. The measurement of oxidation end products could have resulted in further insights in the mechanism of the higher vulnerability in uremia.

Other unmeasured mechanisms leading to higher endothelial cell vulnerability are conceivable. Peroxynitrite exerts, next to the activation of *NRF2* and ARE-driven genes, many other (toxic) effects, such as eNOS uncoupling[7,34] and tyrosine nitration with subsequent inactivation of enzymes or direct nitrosative/oxidative damaging of proteins, lipids or DNA.[20] This could lead to an aggravation of a pre-existing eNOS uncoupling with subsequent O_2^- production, since uremia per se has also been associated with impaired eNOS activity[35] and increased levels of asymmetric dimethylarginine (ADMA), a well-known eNOS inhibitor.[36,37]

Our mRNA expression analyses of *VCAM1*, *ICAM1* and *MMP2* were performed to gain insight in inflammation oriented endothelial cell activation. We did not find increased *VCAM1* or *ICAM1* in the uremic basal conditions, in contrast to previous findings on *VCAM1* and *ICAM1* in uremia.[38,39] This could be partly due to different experimental approaches, such as the timepoint of RNA extraction after exposure, which is rather late for *VCAM1* and early for *ICAM1* mRNA in our study settings. Also, we exposed the HUAECs to both SIN-1 7.5 mM and 10mM to make sure we would trigger oxidative stress-provoked apoptosis, already reported for 10 mM SIN-1.[6] Interestingly, the low dose appeared to trigger apoptosis only for uremia preconditioned cells. The high concentration even triggered occurrence of necrosis, as measured by LDH generation 24 hours after exposure (data not shown). We believe that this generalized cell death explains the decrease in mRNA expression seen in the SIN-1 10mM conditions for *HO-1*, *VCAM-1* and *ICAM-1*. Therefore, we focused mainly on the low concentration for mRNA expression analysis.

Since endothelial cells are among the first to be activated in atherosclerotic degeneration, we focused on these cells, but it would be of equal interest to focus on HO-1 and HO-1 overexpression in uremic monocytes during oxidative stress-dependent atherogenic inflammation.

In conclusion, HUAECs show a higher vulnerability to the oxidative stressor $ONOO^-$ after preconditioning in uremic serum as compared to healthy serum. This cannot be

explained by differential effects of the culture conditions on the expression of the protective enzyme HO-1 or ROS generation. The exact mechanism behind the increased vulnerability remains to be unraveled. Interestingly however, Hemin-induced HO-1 overexpression mitigates this cell vulnerability in uremic conditions by normalizing the apoptotic tendency and by reducing cytoplasmic ROS generation. These findings will stimulate further research for potential therapeutic approaches using HO-1 stimulating agents such as Hemin in uremia.

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Online Supplement 4.1

Detailed materials and methods

Reagents and materials

Hemin, dimethyl sulfoxide (DMSO), gelatin and 3-morpholinosydnonimine (SIN-1) were obtained from Sigma-Aldrich (Bornem, Belgium). Peroxynitrite was purchased from Cayman Chemicals (Bioconnect, Huissen, The Netherlands). Phosphate Buffered Saline (PBS) was purchased from Life Technologies (Gent, Belgium). Endothelial cell media EGM-2 MV bulletkit and EBM2 were purchased from Lonza (Braine-l'Alleud, Belgium). Hemin was dissolved in DMSO to a stock concentration of 2 mM. SIN-1 was dissolved in lukewarm PBS and diluted to a stock concentration of 200 mM.

Serum pools

For the preconditioning of human umbilical artery endothelial cells (HUAECs, Promocell, Bio-Connect, Huissen, Netherlands), serum was pooled from healthy volunteers (HS) (n=10) or from hemodialysis patients (US) (n=40). Blood sampling in dialysis patients was performed immediately prior to dialysis. Biochemical characteristics of the serum pools are given in Table 4.1.

Cell culture conditions

All HUAEC cultures were performed in gelatin-coated flasks or in 6-96 well-plates (Cellstar, Greiner Bio-One, Vilvoorde, Belgium). HUAECs were grown in T75 flasks, supplemented with EGM-2 MV medium 1.5% Pen/Strep and grown till 90% confluence in a 5% CO_2 incubator at 37°C. Cells were then seeded at 50% confluence in 6-96 well plates and grown in basic EBM2-medium supplemented with 30% healthy (HS) or uremic serum (US) for 72 hours, until 90% confluence was reached.

Exposure of HUAEC to $ONOO^-$, SIN-1 and hemin

For the induction of HO-1 and experiments on apoptosis, cells preconditioned in HS and US were exposed to different concentrations of Hemin (25, 50 and 100 μM, dissolved in DMSO), a specific *HO-1* inducer and/or to $ONOO^-$ as a transient oxidative stressor (concentration range 0.1 to 1 mM) or SIN-1 as a source of chronic oxidative stress (7.5 and 10 mM dissolved in PBS). During its decomposition, this water-soluble metabolite of molsidomine releases $O_2^{\cdot -}$ and $NO^{\cdot}$, which spontaneously recombine to form the

highly reactive molecule $ONOO^-$. The use of SIN-1 allows for a more gradual and continuous release of $ONOO^-$ and longer exposure than achievable with $ONOO^-$, which decomposes rapidly.[21] At low concentrations, SIN-1 exerts cytoprotective effects within a narrow range (1-5 mM).[7] However, starting from 7.5 mM, SIN-1 was shown to be cytotoxic, leading to excessive $ONOO^-$ formation and cell toxicity, including induction of apoptosis[7]. Therefore, we compared SIN-1 at 7.5 and 10 mM to simulate increasing oxidative stress, encountered during early atherogenesis, including oxidative stress-mediated cell responses and cell toxicity.
To assess the effect of hemin-induced HO-1 expression on the SIN-1 induced oxidative stress response, cells were first incubated for 5 hours with 50 µM Hemin, followed by exposure to SIN-1 (7.5 or 10 mM). Blanks and controls were analyzed with and without the addition of DMSO to the medium.

RNA extraction and qRT-PCR

To study gene expression, 6 hours after the start of exposure to SIN-1 or Hemin or after exposure to Hemin for 5 hours followed by SIN-1 for another 6 hours, RNA was extracted from trypsinized cell pellets using the RNeasy® microkit (Qiagen, Venlo, The Netherlands) and stored at -80°C until further analysis. (The 6 hour exposure time was chosen based on pilot experiments (data not shown) documenting maximal HO-1 upregulation after 6 hours, the expression returning to baseline levels after 24 hours).
Total RNA (1 µg) was reverse transcribed in 20 µL reactions following a cDNA synthesis protocol using M-MLV transcriptase (Invitrogen, Life Technologies, Gent, Belgium). The cDNA was diluted 10-fold. For *HO-1*, each PCR was carried out in duplicate on a 7500 Fast Real-Time PCR system (Applied biosystems, Gent, Belgium), in a total volume of 12 µL by using 1 µL of the diluted cDNA and 11 µL of primer mix with SYBR Green PCR mastermix (Applied Biosystems, Gent, Belgium). A hot start at 95° C for 5 min was followed by 50 cycles at 95° for 15 sec and 65° for 1 min. Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was determined as a stable endogenous control for each sample. One healthy serum sample was selected as an internal reference for every gene expression analysis. The relative gene expression was calculated by comparing cycle times for target PCR using the following equation: relative gene expression = $2^{-(\Delta CTsample-\Delta CTcontrol)}$ [22].

Reverse transcribed (RT)-PCR products and primer sequences for *HMOX1* and *GAPDH*, purchased from from IDT (Leuven, Belgium), are shown in Table 4.2.
A Taqman Assay (7500 Fast Real-Time PCR System (Life technologies, Gent, Belgium) was used for the RNA expression analysis of Intercellular Adhesion Molecule 1 (*ICAM1*) (Taqman assay ID: Hs00164932), Vascular Cell Adhesion Molecule 1 (*VCAM1*) (Taqman

assay ID: Hs01003372) and Matrix Metalloproteinase 2 (*MMP2*) (Taqman assay ID: Hs01548727). *GAPDH* (Hs02758991) was used as endogenous control gene.

Protein extraction and western blot analysis

Protein analysis was done via western blot analysis, after 8 hours of exposure to SIN-1 or Hemin or after exposure to Hemin for 5 hours followed by SIN-1 for 8 hours. To this end, cells of 3 wells were scraped in 150 µL lysis buffer (containing 100 mM Tris-HCl, pH 6.8, 125 mM NaCl; 1% SDS with addition of 100 mM NaF, 2mM Na_3VO_4 and complete protease inhibitors). The total protein concentration was determined by Bradford quantification.[23] Proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and equal protein amounts were transferred to nitrocellulose. Membranes were incubated with various primary antibodies: anti-HO-1 (rabbit polyclonal Ab, Enzo life sciences ALX-210-116 1/1000), anti-β-Actin (rabbit mAb, Cell signaling technology 4970S 1/1000) and anti-caspase-3 (Rabbit mAb, Cell signaling technologies 9665 1/1000). After adding horseradish peroxidase–conjugated secondary antibodies (Goat anti-Rabbit HRP 1/1000), immunoreactive bands were visualized by ECL and detected on a chemiDoxTM XRS+ Molecular Imager with Imager LABTM software (Bio-rad, Temse, Belgium).

Cell viability and apoptosis

MTT assay

After preconditioning, endothelial cells were exposed to $ONOO^-$ in increasing concentrations from 0.1 to 1 mM. Six hours after exposure, cells were analyzed for viability by the MTT assay. The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble formazan, giving a purple color. MTT was added at 1 mg/mL to the treated cells. After incubation for 2-3 hours, when color conversion occurred, MTT solubilization solution (10% Triton-X 100 in acidic Isopropanol (0,1 N HCL) was added in the same volume as the sample volume and 30 minutes later, colorimetric conversion was read at 550-630 nm on an EL808 Spectrophotometer (Bio-tek, Bad Friedrichshall, Germany).

TUNEL STAINING

After preconditioning, cells were exposed to SIN-1 or Hemin for 8 hours or exposed to Hemin for 5 hours followed by SIN-1 exposure during 8 hours. Endothelial cell apoptosis was estimated by a Cell Death Detection Kit (Roche Applied Science, Brussels, Belgium), according to the manufacturer's instructions, using Flow cytometry analysis (Facs Canto II, BD Biosciences, Erembodegem, Belgium). Mean Fluorescense intensities were

calculated, comprising 90% of the analyzed cell population (only samples with at least 5.000 events/sample were used for analysis). Values were normalized to an internal control HS-precontioned sample. The relative fold increase of the mean Fluorescence intensity is shown in the figures..

Measurement of ROS: Cellrox® and Mitosox®

After preconditioning in black 24- or 96-well plates with clear bottom, cells were exposed to SIN-1 or Hemin for 4 hours or exposed to Hemin for 5 hours followed by SIN-1 exposure during 4 hours.

To detect mitochondrial (mROS) and intracellular, cytoplasmic ROS (cROS) in treated HUAECs, measurements of MitoSOX Red® and CellROX Deep Red® (Invitrogen, Life Technologies, Gent, Belgium) fluorescence were performed by measuring fluorescence intensity in a Flexstation® 3 Microplate reader (Molecular Devices, Berkshire, United Kingdom). After treatment with Hemin and/or SIN-1, cells were incubated with PBS containing 5 µM MitoSOX® for 10 min or 5 µM CellROX® for 30 min at 37°C and 5% CO2. Labeled cells were washed twice with lukewarm PBS. Finally, 250 µL lukewarm EBM2 medium was added for analysis, according to the manufacturer's instructions. Plates were read at excitation/emission wavelengths of 644 nm and 665 nm for CellRox and 510 nm and 580 nm for MitoSox.

Statistics

For all experiments, comparisons were performed by nonparametric ANOVA (Wilcoxon Two-Sample test). Differences were considered significant at $P<0.05$.

CHAPTER 5

HO-1 functional promoter polymorphisms and clinical outcomes in patients with and without CKD

CHAPTER 5.1

Association of HO-1 $(GT)_n$ promoter polymorphism and cardiovascular disease: a reanalysis of the literature

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Abstract

Background

Heme-Oxygenase 1 (HO-1), an inducible heme-degrading enzyme, has anti-atherogenic effects through its enzymatic end-products. HO-1 gene expression is modulated by a $(GT)_n$ repeat polymorphism in the promoter region. Shorter repeats with $(GT)_n<25$ are associated with higher inducibility and activity of HO-1.

Methods and results

We performed a systematic review of all literature from 1997 to 2013 studying the association of the HO-1 (GT)n repeat length and cardiovascular disease. Based on pre-defined criteria (patient characteristics, genotype data format, allelic distribution, repeat length cutoff) 41 articles were selected. Patients were redistributed into 4 homogeneous subpopulations: cardiovascular diseased patients (CVD), non-cardiovascular diseased patients (nonCVD), 'controls' with unknown cardiovascular status (unspecified) and children <20 years (unselected). Genotype distributions [homozygous short (SS) or long (LL), heterozygous (SL)] of the four patient categories were compared and Odds ratios for CVD were calculated using logistic regression analysis. Overall, the proportion of the SS genotype was lower in CVD as compared to nonCVD and unspecified. The Odds for cardiovascular disease was highest in patients carrying the LL-genotype (OR LL vs. SS 1.769 (1,594-1,963)). Furthermore, genotype distribution differs between Caucasians and Asians, the latter having a much higher proportion of the SS-genotype (22% vs. 11%).

Conclusions

This review of the available literature on the epidemiological association between the HO-1 (GT)n repeat polymorphism and cardiovascular disease supports the presumed protective effects of HO-1. The second but probably even more relevant finding of our review is that racial disparities in HO-1 $(GT)_n$ repeat length distribution exist and may influence the associations of the genotype with CVD status.

Introduction

Heme-oxygenase-1 (HO-1) is an inducible, stress-responsive enzyme responsible for the rate-limiting enzymatic degradation of heme to free ferrous iron, carbon monoxide (CO) and biliverdin,[1] the latter being rapidly converted by biliverdin reductase to bilirubin. Each of these enzymatic end-products exerts anti-oxidative, anti-inflammatory and anti-apoptotic effects through different mechanisms.[2,3] HO-1 can be induced by a variety of agents including heme, cytokines and endotoxins and its activity is influenced by genetic factors. In the promoter region of the HO-1 gene, located on chromosome 22q12,[4] several functional polymorphisms have been found: a T(–413)A single nucleotide polymorphism (SNP rs2071746)[5,6] and a $(GT)_n$ dinucleotide repeat polymorphism. Data on the T(-413)A SNP are scarce and conflicting.[5-7] The $(GT)_n$ polymorphism, however, has been studied extensively. It consists of variable lengths of purine–pyrimidine alternating repeats. Longer repeats acquire the potential to assume Z-DNA conformation, a lefthanded double-helix structure. This Z-DNA conformation negatively affects transcriptional activity.[8] Longer repeats result therefore in lower HO-1 expression and activity, as confirmed by luciferase promoter constructs and transient transfection assays in different cell lines.[9,10]

The protective properties of HO-1 have been extensively studied in *in-vitro* and animal models of ischemia-reperfusion injury and acute kidney injury (AKI).[11-13] These findings are corroborated by clinical associations between the HO-1 genotype and outcome of organ transplantation and AKI. The present review focuses on atherosclerosis, another field in which a prominent role of HO-1 has been recognized.[14,15]

Wang et al. first described the presence of HO-1 in human atherosclerotic plaques whereas no HO-1 expression was seen in normal arteries.[16] Animal studies show a role of HO-1 in the initiation of atherogenesis by protecting against plaque formation.[17,18] Moreover, HO-1 is found to be implicated in plaque stabilization in the later stages of atherosclerosis.[14,19,20]. Based on these mechanistic findings, many authors have studied the association between the above-mentioned $(GT)_n$ repeat length polymorphism and the presence of cardiovascular disease (CVD) in different populations.[21-31] The published results, however, are conflicting. One of the problems with the interpretation of these data is the great diversity in study populations (race, co-morbidity…), genotyping methodology and end-point definitions. The aim of the present review was to bring together all available data on HO-1 $(GT)_n$ repeat polymorphisms in clinical populations, to select high-quality information based on strict methodological criteria and to dissect homogeneous subpopulations to study their $(GT)_n$ repeat distributions with regard to the presence or absence of established cardiovascular disease.

Materials and methods

Search strategy

A MEDLINE literature search of papers in all languages published between January 1997 and August 2013 was performed using the MESH-terms 'HO-1' OR 'heme oxygenase-1' together with (AND) 'repeat polymorphism' OR 'GT polymorphism' OR 'polymorphism'. Reviews, studies on HO-1 SNP, *in-vitro* experiments and animal model investigations were excluded. Full text revision and screening of the reference lists was performed but did not reveal additional papers. After excluding 4 articles of which no full text version was available and 1 of 2 articles describing the same patient population,[10,32] 102 papers were eligible for detailed evaluation and further selection (flow diagram in Figure 5.1.1).

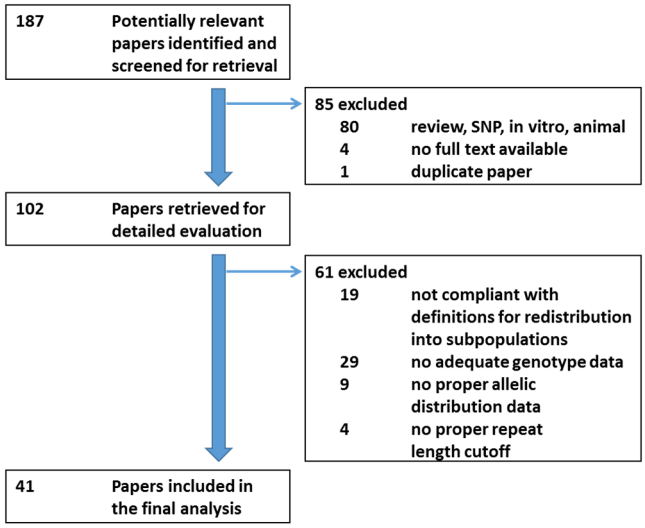

Figure 5.1.1 Flow Diagram of the performed literature research and article selection.

Eligibility selection criteria (flow diagram, Figure 5.1.1)

Compliance with definitions for redistribution into four homogeneous subpopulations

In order to define homogeneous subpopulations with regard to CVD status, four distinct groups were identified. The **cardiovascular patient group** (**CVD**) encompasses all patients with established CVD as explicitly verified by further examinations a.o. ECG, angiography, exercise test, MIBI scintigraphy or sonography (carotids or ankle/brachial index). The **non-cardiovascular patient group** (**nonCVD**) gathers all patients free of cardiovascular disease as explicitly verified by the abovementioned examinations. A

third group contains patients in whom cardiovascular status cannot be judged since no explicit cardiovascular data are reported. This group is called "**unspecified**" and pools control populations of studies on non-cardiovascular topics (healthy volunteers, organ or bone marrow donors, general population). Finally, a limited number of papers provided information on neonates and children <20 years of age, a population that can be considered **unselected** from a CVD viewpoint.
Populations not complying with one of the four mentioned definitions were excluded from the analysis. These comprised patients with another diseased condition (for excluded references, see online supplement Table 5.1.1).

Availability of adequate genotype data (format)
In all papers genotype data were recalculated using the following format: one $(GT)_n$ cutoff dividing alleles into two groups (short, long) and genotypes into three groups (homozygous short (SS), heterozygous (SL), homozygous long (LL)). Papers in which only allelic distribution was reported or in which genotype data were incomplete or based on two or more $(GT)_n$ cutoffs, rendering recalculation into the above-mentioned format impossible, were excluded from the analysis (Online Supplement Table S5.1.1).

Proper allelic distribution
The $(GT)_n$ repeat length is determined by PCR using primers that flank both ends of the repeat sequence, and thus generate a DNA fragment of which the length depends on the number of repeats in the microsatellite. These fragments are analyzed by electrophoresis using a size standard to determine the repeat number. The HO-1 $(GT)_n$ repeat length distribution has consistently been shown to be bimodal with 23 and 30 repeats as the most common repeat lengths. In this way, the allelic distribution is a direct reflection of the methodology of the sequencing process. Some studies described 22 and 29 repeats as most frequent, probably reflecting a minor erroneous shift in the fragment-analysis, but were corrected afterwards. Given the relevance of the allelic distribution as a parameter of methodological scrutiny, articles with unknown or aberrant allelic distribution were excluded from the analysis (online supplement Table 5.5.1).

Proper repeat length cutoff for the short allele
There is no consensus on the optimal cutoff for the $(GT)_n$ repeat length in the HO-1 promoter. Luciferase activity assays and mRNA expression analysis in aortic smooth muscle cells and peripheral blood monocytes shows higher expression in GT repeat lengths with less than 26 repeats.[10,33] General consensus accepts cutoffs between 24 and 27, since distributions within this range are not significantly different.[34] Papers in which higher or lower cutoffs were used, were excluded from the analysis (online supplement Table S5.1.1).

Table 5.1.1 Details of the 41 studies included in the final analysis. (for references, see Online Table S5.1.2)

Author, year	Population description	Age	Country	Ethnicity	N	Cutoff S <	Allelic distribution	Group
	Studies with cardiovascular end-points (controls and cases eligible for inclusion in review)							
Chen Mu 2012	No CAD or PAD (exclusion by anamnesis, clinical examination and ECG)	59.9±10	China	Asian	2298	25	23/30	nonCVD
	CAD (≥1 Coronary Artery > 50% stenosis or history for event/surgery)	60±10	China	Asian	2298	25	23/30	CVD
Chen YH 2008	No CAD (excluded on angio, <20% stenosis) or history/clinic of atherosclerosis in other vascular beds	67±10	Taiwan	Asian	664	27	23/30	nonCVD
	CAD (>75% stenosis on angio, history of AMI or CABG)	69±9	Taiwan	Asian	322	27	23/30	CVD
Chen YH 2004	CAD patients with successful single PCI for critical stenosis (>60%)	68±9	Taiwan	Asian	323	26	23/30	CVD
Dick 2005	PAD patients Fontaine ≥ IIb scheduled for revascularization	69 (60-76)	Austria	Caucasian	472	25	23/30	CVD
Endler 2004[23]	CAD patients (diagnose by angio, clinic and/or history of event/intervention)	62 (55-71)	Austria	Caucasian	438	25	23/30	CVD
	No CAD (exclusion by angio ± scintigraphy); No further exclusion of CVD	58 (48-67)	Austria	Caucasian	211	25	23/30	nonCVD
Exner 2001[31]	PTA patients Fontaine ≥ IIb, no stent, Fem or Pop Artery	69 (60-75)	Austria	Caucasian	96	25	24/29 Corrected	CVD
Funk 2004	No CVD (excluded by history and/ clinical examination)	47 (40-59)	Austria	Caucasian	398	25	22/29	nonCVD
	Ischemic stroke event or TIA	69 (59-78)	Austria	Caucasian	399	25	22/29	CVD
Gregorek 2012	AAA (diagnose by US or CT), CVD in ± 15% of group	69 (62-73)	Croatia	Caucasian	117	25	23/30	unspecified
	No CVD (excluded by US, ECG and/or ABI) or AAA (excluded by US or CT)	64 (57-73)	Croatia	Caucasian	117	25	23/30	nonCVD
Holweg CT 2005	Heart donors	Approx. 27	The Netherlands	Caucasian	253	27	23/30	unspecified
	Heart Tx recipients (origin heart disease: Ischemic or dilated cardiomyopathy)	Approx. 48	The Netherlands	Caucasian	296	27	23/30	CVD
Kaneda 2002	CAD (>75% stenosis); other CVD not investigated	63±0,5	Japan	Asian	298	27	23/30 corrected	CVD
	No CAD (angiography, cutoff not mentioned) Other CVD not excluded	58±0,7	Japan	Asian	279	27	23/30	unspecified
Lublinghoff 2009	No CAD (coronary stenosis < 20%), other CVD not excluded	58±11	Germany	Caucasian	693	25	22/29	nonCVD
	CAD (CAD if 1/15 coronary segments >20% stenosis) **	64±10	Germany	Caucasian	2526	25	22/29	CVD

Table 5.1.1 (continued)

Author, year	Population description	Age	Country	Ethnicity	N	Cutoff S <	Allelic distribution	Group
Wu 2010	No Carotid atherosclerosis, excluded by ECCA-US (IMT <1.0mm and no plaque) Other CVD not excluded	approx. 65	Taiwan	Asian	420	27	23/30	unspecified
	Carotid atherosclerosis, by ECCA-US (IMT or plaque (> 50% wall thickening))	approx. 55	Taiwan	Asian	367	27	23/30	CVD
Wu 2011	General, arsenic-exposed, population (Study aim: Hypertension, CV outcome)	58.7±10	Taiwan	Asian	894	27	23/30	unspecified
Schillinger 2002	No CVD (excluded by med history, clinic exam and/or ultrasonography)	51 (40-68)	Austria	Caucasian	62	25	23/30	nonCVD
	CVD patients with either 2- or 3vessel CAD or PAD Fontaine ≥ IIb	71 (62-78)	Austria	Caucasian	210	25	23/30	CVD
Schillinger 2004	PTA Fontaine≥ IIa (balloon angioplasty, stenting or angiography) 50% have also other CVD.	approx. 70 (60-75)	Austria	Caucasian	381	25	23/30	CVD
Tiroch 2007	Symptomatic CAD (Patients undergoing PCI+BMS)	65.7±10	Germany	Caucasian	1807	25	23/30	CVD
	Studies with non-cardiovascular end-points (only controls eligible for inclusion in review)							
Alexeeff 2008	Elderly healthy men [study aim: ozone exposure and lung function]	69±7.2	USA, 98% white	Caucasian	971	25	23/30	unspecified
Bozkaya 2010	Newborns, random selection	0	Turkey	Caucasian	152	24	23/30	unselected
Buis 2008	Controls: Liver transplant donors, general pop (73 % CVA) [cases: Liver transplant recipients]	46 (35-55)	The Netherlands	Caucasian	308	25	22/29	unspecified
Chang 2012	Controls [cases: Psoriasis patients]	46±10	China and Taiwan	Asian	542	27	23/30	unspecified
Choi 2012	Type 2 diabetes patients, otherwise in good health [Study aim: oxidative stress, glycemic control and complication development]	59±10	China	Asian	418	25	23/30	unspecified
Courtney 2007	Kidney donors of donor/recipient pairs [Study aim: Tx outcome]	37±17	Europe	Caucasian	681	25	23/30	unspecified
Exner 2004	Kidney donors [Study aim: Tx allograft outcome]	48 [38-58]	Europe	Caucasian	101	25	23/30	unspecified
Fu 2007	Controls: healthy smokers, COPD excluded by chest CT [Cases: COPD patients]	70±7	China	Asian	266	25	22/29, 30	unspecified

Table 5.1.1 (continued)

Author, year	Population description	Age	Country	Ethnicity	N	Cutoff S <	Allelic distribution	Group
Geuken 2005	Controls: Liver donors [Cases: liver tx patients]	39 (25-60)	The Netherlands	Caucasian	37	25	22/29	unspecified
Guénégou 2006	General population [Study aim: Lung function decline]	37±7	Europe	Caucasian	749	27	23 30/38	unspecified
Hong CC 2007	Postmenopausal women [Cases: Breast cancer]	50-74	USA	Caucasian	491	25	23/30	unspecified
Hu 2010	Controls: Cancer-free males [Cases: Oesophageal cancer]	58	Taiwan	Asian	264	25	23/30	unspecified
Jiraskova 2012	Clinically Healthy controls [Cases: sporadic colorectal cancer]	49±11	Czech Republic	Caucasian	986	27	23/30	unspecified
Kanai 2003	Controls: adolescents [Cases: neonatal hyperbilirubinemia]	-	Germany	Caucasian	89	27	23/30	unselected
Kanai 2003	Controls: adolescents [Cases: Kawasaki disease]	24±19	Japan	Asian	122	27	23/30	unselected
Katana 2010	Kidney Donors of donor/recipient pairs [Study Aim: renal Tx outcome]	53±14	Greece	Caucasian	162	27	23/30	unspecified
Katana 2011	Healthy volunteers [Study aim: genotype distribution in general population]	35 (18-60)	Greece	Caucasian	250	25	23/30	unspecified
Lo 2007	Controls [Cases: gastric adenocarcinoma patients]	51±17	Taiwan	Asian	250	26	23/30	unspecified
Murakami 2012	Controls: asbest exposure [Cases: Malignant Mesothelioma]	67 (48–84)	Japan	Asian	43	24	23/30	unspecified
Okamoto 2006	Controls: free of clinical manifest disease [Cases: malignant melanoma]	48±14	Austria	Caucasian	398	25	22/29	unspecified
Rueda 2007	Blood bank and bone marrow donors [Cases: rheumatoid arthritis]	45±12	Spain	Caucasian	846	26	23/30	unspecified
Song 2009	Controls, no known disease [Cases: DM type 2]	48±12	China	Asian	1581	25	22/30	unspecified
Ullrich 2005	Controls: Heart donors [Cases: Cardiac allograft vasculopathy]	32±9	Austria	Caucasian	152	25	23/30	unspecified
Wagener 2008	Controls: Anonymous healthy blood donors [Cases: Rheumatoid arthritis]	None reported	The Netherlands	Caucasian	273	25	23/30	unspecified
Yamada 2000	smokers, 38% CVD [Study aim: pulmonary emphysema]	67±0,9	Japan	Asian	201	25	23/30	unspecified

AAA abdominal aorta aneurysm, ABI ankle/brachial pressure Index, AMI acute myocardial infarction, Angio Angiography, Approx. Approximately, CABG coronary artery bypass graft, CAD Coronary artery disease, COPD chronic obstructive pulmonary disease, CT computed tomography, CVD cardiovascular disease, DM Diabetes Mellitus, ECCA-US Ultrasound of extracranial carotid artery, ECG electrocardiogram, Fem Femoral, IMT intima-media thickness, N number, PAD peripheral artery disease, PCI percutaneous coronary intervention, Pop popliteal, PTA percutaneous transluminal angioplasty, TIA Transient Ischemic attack, Tx transplantation, US ultrasound. For references of included articles, see Online Supplemental Table 5.1.2.

Data extraction

Author name, year of publication, description and selection of the studied population (controls and cases), mean/median age (as reported in the reference), country or region, ethnicity, cutoff for the short allele and allelic distribution were extracted from the selected papers and reported in Table 5.1.1.
For the purpose of this review patient data were then redistributed into above-mentioned categories: **CVD, nonCVD, unspecified, unselected** (Table 5.1.1). For each of these subpopulations genotype frequencies, extracted or calculated from the individual trials, were pooled together for further statistical analysis. The bibliographic search, paper selection and data extraction were conducted independently by 2 authors (KD, PM) and disagreements were resolved by consensus for all data.

Statistical analysis

$(GT)_n$ repeat distributions in each of the predefined patient subpopulations are reported as absolute and relative frequencies of the SS-, SL- and LL-genotypes. In a separate analysis, SS- and SL-genotypes were taken together as S-carriers. Two by two comparisons of genotype distributions between the CVD population on the one hand and the nonCVD, unspecified or unselected populations on the other were done using Mantel-Haenszel Chi Square statistics (MH) for association (SS, SL, LL) and Logistic Regression analysis reported as odds ratios (ORs) (SS vs. LL and S-carrier vs. LL). In a secondary analysis, Caucasian and Asian patients were analyzed separately. All statistical analyses were performed using the statistical package SAS version 9.3 (SAS Institute, Cary, NC, USA).

Results

Selected articles

Forty-one papers fulfilled the stepwise selection criteria for inclusion in the analysis mentioned above (flow diagram, Figure 5.1.1). Table 5.1.1 displays the individual articles. Sixteen papers had cardiovascular end-points. For the present analysis, their populations contributed to the CVD, nonCVD and unspecified patient groups. Twenty-five studies reported on polymorphism distributions in non-cardiovascular disease states. The study end-point and/or the case population of these studies are mentioned between brackets in Table 5.1.1. The control groups of these cohort studies were included in the present analysis and contributed to the unspecified and unselected patient groups. Total overall numbers of the populations were n=10634 for CVD, n=4443 for nonCVD, n=12010 for unspecified and n=363 for unselected. Within each of these subpopulations the Hardy-Weinberg equilibrium was fulfilled.

Overall analysis (Table 5.1.2a and Online Table 5.1.3a)

CVD vs. nonCVD

The proportion of the SS-genotype is lower in the CVD compared to the nonCVD population (13,3% vs. 18,9%, MH *P*<0.0001). The OR for CVD in the LL vs. SS-genotype is 1,769 (1,594-1,963).

CVD vs. unspecified

The proportion of the SS-genotype is lower in the CVD as compared to the unspecified population (SS 13,3% vs. 17,4% MH *P*<0.0001). The OR for CVD in the LL vs. SS-genotype is 1,478 (1,366-1,601).

CVD vs. unselected

The proportion of the SS-genotype is lower in the CVD as compared to the unselected population (SS 13,3% vs. 15,2% MH P 0.006) with a non-significant OR for CVD in the LL vs. SS-genotype of 0,995 (0,724-1,347).

Analysis confined to Caucasian population only (Table 5.1.2b and Online Table 5.1.3b)

CVD vs. nonCVD

There was no difference in the proportion of the SS-genotype in the Caucasian CVD as compared to the Caucasian nonCVD population (SS 10,2% vs. 9,7% MH P 0.38). The OR for CVD in the LL vs. SS-genotype is 0.969 (0.796-1.179).

CVD vs. unspecified

The proportion of the SS-genotype is lower in the Caucasian CVD as compared to the Caucasian unspecified population (SS 10,2% vs. 12,3% MH *P*<0.0001). The OR for CVD in the LL vs. SS-genotype is 1,179 (1,098-1,266).

CVD vs. unselected

The proportion of the SS-genotype is lower in the Caucasian CVD as compared to the Caucasian unselected population (SS 10,2% vs. 12,0% MH P 0.01) with a non-significant OR for CVD in the LL vs. SS-genotype of 1,028 (0,67-1,526).

Analysis confined to Asian population only (Table 5.1.2c and Online Table 5.1.3c)

CVD vs. nonCVD

The proportion of the SS-genotype is lower in the Asian CVD as compared to the Asian nonCVD population (SS 19,3% vs. 23,5% MH P 0.33). The OR for CVD in the LL vs. SS-genotype is 1,2671 (1,100-1,460).

CVD vs. unspecified

The proportion of the SS-genotype is lower in the Asian CVD as compared to the Asian unspecified population (SS 19,3% vs. 24,2% MH P 0.003). The OR for CVD in the LL vs. SS-genotype is 1,242 (1,097-1,406).

CVD vs. unselected

The proportion of the SS-genotype is only slightly lower in the Asian CVD as compared to the Asian unselected population (SS 19,3% vs. 21,3% MH P 0.235) with a non-significant OR for CVD in the LL vs. SS-genotype of 0,787 (0,518-1,195).

Tabel 5.1.2 Distribution of (GT)n genotype (SS, SL, LL) for the overall (a), Caucasian (b) and Asian (c) subpopulation.

ALL	SS	SL	LL	Total	%SS	%SL	%LL	OddsRatio LL vs SS
CVD	1415	4866	4353	10634	13,3	45,8	40,9	
nonCVD	839	2145	1459	4443	18,9	48,3	32,8	1,769 (1,594-1,963)
Unspecified	2093	5562	4355	12010	17,4	46,3	36,3	1,478 (1,366-1,601)
Unselected	55	138	170	363	15,2	38,0	46,8	0,995 (0,724-1,347)
Total	4402	12711	10337	27450	16	46	38	

CAUCASIAN	SS	SL	LL	Total	%SS	%SL	%LL	OddsRatio LL vs SS
CVD	719	2916	3391	7026	10,2	41,5	48,3	
nonCVD	144	636	701	1481	9,7	42,9	47,3	0.969 (0.796-1.179)
Unspecified	834	2984	2943	6761	12,3	44,1	43,5	1.179 (1.098-1.266)
Unselected	29	79	133	241	12,0	32,8	55,2	1,028 (0,67-1,526)
Total	1726	6615	7168	15509	11	43	46	

ASIAN	SS	SL	LL	Total	%SS	%SL	%LL	OddsRatio LL vs SS
CVD	696	1950	962	3608	19,3	54,0	26,7	
nonCVD	695	1509	758	2962	23,5	50,9	25,6	1.267 (1.100-1.460)
Unspecified	1247	2524	1388	5159	24,2	48,9	26,9	1.242 (1.097-1.406)
Unselected	26	59	37	122	21,3	48,4	30,3	0,787 (0,518-1,195)
Total	2664	6042	3145	11851	22	51	27	

Proportions were calculated using table analysis, Conditional Odds ratios with Wald confidence intervals with logistic regression analysis. ORs for CVD in LL vs. SS groups are reported in last column. CVD cardiovascular disease.

Discussion

The major finding of this systematic review of the epidemiological literature on HO-1 $(GT)_n$ promoter polymorphisms is that the short repeat SS-genotype is represented in a lower proportion of patients with established cardiovascular disease as compared to a population without cardiovascular disease or control patients of whom the cardiovascular status was not explicitly assessed. Consequently, the odds for cardiovascular disease was higher in patients carrying the long repeat LL-genotype as compared to their heterozygous or homozygous short allele carriers. The second but probably even more relevant finding of our review is that racial disparities in HO-1 $(GT)_n$ repeat length distribution exist and may confound the association of the genotype with cardiovascular disease status.

Heme oxygenase-1 is an important anti-atherosclerotic enzyme, exerting anti-oxidative and anti-inflammatory effects through the degradation of heme, thereby releasing free iron, CO and bilirubin. Each of these end-products exerts direct and indirect anti-atherogenic functions.[17,19] Indeed, biliverdin and bilirubin are well known scavengers for reactive oxygen species (ROS). CO influences signaling pathways resulting in induction of anti-inflammatory and inhibition of inflammatory and apoptotic processes, inhibition of platelet aggregation, relaxation of smooth muscle cells and reduction of endothelin-1 and platelet derived growth factor in the endothelial cells of the arterial wall.[35-37] Free iron is considered to rapidly induce the formation of ferritin and an ATPase pump that actively removes intracellular iron from the cell. The resulting modulation of the intracellular iron store and increased iron efflux are believed to be the mechanisms by which HO-1 confers resistance to oxidative stress.[38]

In support of these advantageous effects of HO-1, animal studies show that the absence of HO-1 leads to accelerated atherosclerosis and that overexpression of the enzyme protects against plaque formation.[17,18] OxLDL and ROS, such as peroxynitrite, generated in early atherosclerotic lesions induce HO-1.[39,40] The enzyme consequently mitigates the initial phase of atherogenesis by decreasing lipid peroxidation, reducing apoptosis in endothelial cells and reducing inflammation, more specifically through decreased MCP-1 generation and subsequent monocyte recruitment.[41,42] In established atherosclerosis, HO-1 was found to stabilize plaques, preventing the development of vulnerable plaques which are more prone to complications.[14,20,43] It is of note that statins, well-known therapeutic agents in the treatment of atherosclerosis, were found to exert their effects partly through the induction of HO-1.[44]

Several gene promoter polymorphisms have been found to influence HO-1 activity and particularly the $(GT)_n$ repeat length polymorphism is considered of substantial functional importance. Shorter repeat lengths, which are associated with higher HO-1 activity, would contribute to protection against atherogenesis. In a study by Kral et al.[45] homozygous LL-genotype carriers had greater coronary artery atherosclerotic burden and more pronounced plaques expressed by higher percentages of atheroma volume.

Furthermore, they showed more plaques with a necrotic core and a higher occurrence of thin fibrous cap atheroma. This is consistent with the animal findings.[17]

However, while in vitro and animal data clearly show the protective function of HO-1 in atherogenesis, clinical results and genotype data remain conflicting. This may at least partly be explained by the limited sample size of the studied populations in some papers. On the other hand, published data differ in study population characteristics (race, co-morbidity…), genotyping methodology and end-point definitions. In the present review the power issue was overcome by pooling all available data on HO-1 $(GT)_n$ repeat polymorphisms in clinical populations published between January 1997 and August 2013. Proper genotyping methodology was ascertained by the application of rigorous selection criteria. The major novelty of this review, however, is that after careful selection of eligible papers, the populations were redistributed in four distinct groups of patients in order to mitigate study heterogeneity.

As mentioned above, the overall conclusion of our analysis corroborates the fundamental and mechanistic data on HO-1. Indeed, the LL-genotype (lowest HO-1 activity) is found to be overrepresented in patients with established cardiovascular disease (see Table 5.1.2a and online Table 5.1.3a).

However, when analyzing the data in more detail, some additional interesting issues appear.

First, HO-1 $(GT)_n$ distribution clearly differs according to race. In the pooled Caucasian populations we found an overall proportion of the SS-genotype of 11%, whereas in the Asian population this reaches up to 22% (see Table 5.1.2b-c). The difference is even more pronounced when considering S-carriers (SS and SL) (see Online Table 5.1.3b-c). Since no single studies comprising Asian and Caucasian subpopulations together have been performed, one might argue that the found differences are spurious and due to coincidental methodological differences between trials. However, since reported allelic distribution and sequencing methods do not differ between the different manuscripts, this is highly unlikely. Comparative epidemiological literature on incidences and prevalences of cardiovascular disease between Caucasian and Asian populations are scarce. One study of Howard et al., conducted in the United States, showed lower age-adjusted mortality rates for ischemic heart disease in Asian subjects as compared to non-Hispanic white subjects.[46] Another study by Slater et al., following 4186 patients of white, Hispanic, black or Asian origin after undergoing a PCI shows the lowest 2-year mortality in the Asian subpopulation.[47] It is tempting to speculate that the noted differences in HO-1 genotype distribution between the Caucasian and Asian populations contribute to differences in cardiovascular disease outcomes. However, it should be acknowledged that significant differences in lifestyle and diet between these ethnic groups may confound the apparent role of disparities in genotype distribution. The same holds true for traditional risk factors such as smoking, dyslipidemia and diabetes. Unfortunately, from the fact that no systematic information on these confounders is provided in the studies included in our review, it is impossible to

formally analyze the data in this respect and further research in this area is warranted. Nevertheless, the most important conclusion from our finding of differential racial HO-1 genotype distribution is that pooling data from the two populations, as reported in Table 5.1.2a and Online Table 5.1.3a can be problematic. Indeed, in the reviewed papers the overall number of Asian nonCVD patients is higher than Caucasian nonCVD patients, while the opposite is true in the CVD population. As such, the epidemiological evidence supporting the anti-atherogenic effect of the HO-1 polymorphism is clearly overinterpreted in the pooled analysis (Table 5.1.2a and Online Table 5.1.2a). Nevertheless, even after stratifying for ethnicity, LL carriers have increased, albeit modest, odd ratios for cardiovascular disease (see Tables 5.1.2b-c and Online Tables 5.1.3b-c).

Second, redistribution of the patients resulted in four distinct subpopulations. This approach clearly improved homogeneity of the studied groups. Indeed, it can be assured that the patients of CVD group have documented CVD. Alternatively, in patients of the nonCVD group CVD disease was actively ruled out. In some studies only the absence of coronary artery disease (CAD) is ascertained for attribution to the control population while atherosclerosis in other vascular beds was not actively investigated. Being fully aware of these limitations, the unspecified group was created to separately pool control populations in whom cardiovascular disease was not actively ruled out. This is by definition the most heterogeneous subpopulation. Nevertheless, even within the CVD and nonCVD subpopulations substantial residual heterogeneity prevails, since investigators have used divergent criteria to define CVD. For instance, Lublinghoff et al. used a cutoff of >20% luminal stenosis as the definition of CAD, whereas most studies only considered >75% luminal stenosis as relevant atherosclerotic disease.[34] Furthermore, there are remaining issues such as the lack of matching for age and sex in some case-control studies and the differences in background comorbidities, for which it was not possible to properly control the data. As ageing is a well-known and important risk factor for CVD, studying cases and controls from different age obviously causes potential selection bias. As mentioned earlier, background comorbidities such as the conventional cardiovascular risk factors are expected to be of potential influence on the development of cardiovascular disease as well as on the activity or efficacy of HO-1. Smoking, obesity and diabetes, all associated with increased oxidative stress, were found to play an important role. Of note, Chen et al., Dick et al. Kaneda et al. and Wu et al. found that HO-1 protects against adverse cardiovascular events only in the presence of conventional risk factors or increased oxidative stress.[22,27,28,30]

Finally, it is clear that the CVD, nonCVD and unspecified populations in the respective papers and the current review of those papers comprises a population of adult patients in whom natural selection most probably has already occurred. In order to gain more insights in racial disparities and associations of the genotype with cardiovascular outcomes, the evaluation of large cohorts of unselected populations, such as newborns

could be helpful. So far however, only limited data are available, as can be judged from the unselected subpopulation described in Table 5.1.2 and Online Table 5.1.3. The mere absence of significant findings in this subpopulation is in our view of limited relevance given the very low power of this subanalysis. Further research in this field is warranted.

In conclusion, this review of the available literature on the epidemiological association between HO-1 (GT)n promoter polymorphism and cardiovascular disease supports the presumed protective effects of HO-1 enzymatic activity. The data point however to variations of genotype distribution between ethnic groups, which should be taken into account when interpreting data from published studies. For further investigation of genotype-phenotype associations, a better insight in genotype distributions of unselected populations of neonates and young adolescents is warranted.

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Online Supplement 5.1.1

Tabel S5.1.1 Excluded articles.

Reason exclusion	not compliant with definitions for redistribution in subpopulations

1. Walther M, De CA, Aka P, Njie M, Amambua-Ngwa A, Walther B, Predazzi IM, Cunnington A, Deininger S, Takem EN, Ebonyi A, Weis S, Walton R, Rowland-Jones S, Sirugo G, Williams SM, Conway DJ. HMOX1 gene promoter alleles and high HO-1 levels are associated with severe malaria in Gambian children. *PLoS Pathog* 2012;8(3):e1002579.
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Reason exclusion no adequate genotype data format

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Table S5.1.3 Distribution of (GT)n genotype (S-carrier, LL) for the overall (a), Caucasian (b) and Asian (c) subpopulation.

A. ALL	**S - carrier**	**LL**	**Total**	**% S - carrier**	**% LL**	**Odds Ratio LL vs. S-carrier**
CVD	6281	4353	10634	59,1	40,9	
nonCVD	2984	1459	4443	67,2	32,8	1,417 (1,317-1,526)
Unspecified	7655	4355	12010	63,7	36,3	1,218 (1,155–1,285)
Unselected	193	170	363	53,2	46,8	0,787 (0,638-0,971)
Total	17113	10337	27450	62	38	

B. CAUCASIAN	**S-carrier**	**LL**	**Total**	**% S-carrier**	**% LL**	**Odds Ratio LL vs. S-carrier**
CVD	3391	3391	7026	51,7	48,3	
nonCVD	780	701	1481	52,7	47,3	1,038 (0,928-1,161)
Unspecified	3818	2943	6761	56,4	43,5	1,210 (1,069-1,150)
Unselected	108	133	241	44,8	55,2	0,758 (0,584-0,980)
Total	8341	7168	15509	54	46	

C. ASIAN	**S-carrier**	**LL**	**Total**	**% S-carrier**	**% LL**	**Odds Ratio LL vs. S-carrier**
CVD	2646	962	3608	73,34	26,7	
nonCVD	2204	758	2962	74,41	25,6	1,057 (0,946-1,181)
Unspecified	3771	1388	5159	73,10	26,9	0,988 (0,897-1,087)
Unselected	85	37	122	69,7	30,3	0,835 (0,568-1,251)
Total	8706	3145	11851	73	27	

Proportions were calculated using table analysis, Conditional Odds ratios with Wald confidence intervals with logistic regression analysis: ORs for CVD in LL vs. SS groups are reported in last column. Abbreviations are: CVD cardiovascular disease.

CHAPTER 5.2

Heme oxygenase-1 promoter polymorphisms and cardiovascular disease in dialysis patients

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Submitted

Abstract

In dialysis, oxidative stress is widely recognized as a key player in atherosclerosis. Heme oxygenase-1 (HO-1) is an antioxidative and antiatherosclerotic enzyme. HO-1 expression and activity is modulated by a $(GT)_n$ repeat polymorphism and a T(-413)A single nucleotide polymorphism. Shorter repeats with $(GT)_n$<25 and the A allele are associated with higher inducibility and activity of HO-1. We studied the association between the HO-1 polymorphisms and cardiovascular disease (CVD) in 205 dialysis patients. Demographic, biochemical and clinical data were retrieved from patient files. The HO-1 $(GT)_n$ repeat length long (L) and short (S) alleles were defined as ≥25 and <25 repeats based on literature data. SNP polymorphism genotypes were classified as AA, AT and TT. The $(GT)_n$ repeat polymorphism was distributed as follows: SS 8,6%; SL 36%; LL 55%. We compared 104 CVD patients with 101 nonCVD patients. The proportion of SS/LL carriers was 12%/48% in the nonCVD group and 5%/62% in the CVD group. Univariate logistic regression analysis showed an odds ratio of 3,148 [1,039 – 9,541] for CVD in the LL vs. SS carriers. After multivariate analysis this effect lost significance in favor of diabetes, higher age and dialysis duration. In our study population, no significant association between the T(-413)A SNP polymorphism and CVD was found. Our findings are in line with a role of the HO-1 $(GT)_n$ repeat in the occurrence of CVD in a dialysis population. Dialysis patients with the LL genotype have an increased risk for cardiovascular disease compared to those with the SS genotype.

Introduction

In dialysis patients the prevalence of cardiovascular disease (CVD) is high.[1] Chronic kidney disease (CKD) is characterized by accelerated atherosclerosis, and recent data indicate that this process already begins with minor renal insufficiency.[2] Traditional risk factors such as diabetes, arterial hypertension and dyslipidemia are insufficient to explain the high rate of atherosclerosis in CKD patients.[3] Therefore recent research shifted towards non-traditional risk factors, including anemia, increased vascular calcification, endothelial dysfunction, inflammation and oxidative stress, among others.[4] Heme oxygenase-1 (HO-1), an inducible enzyme with antioxidative and anti-inflammatory properties, has been demonstrated to be a key player in protection against experimental atherosclerosis.

HO-1 catabolizes heme into three breakdown products: carbon monoxide, biliverdin and free iron.[5] These end products have anti-inflammatory, anti-apoptotic and antioxidative effects. The induction of endogenous HO-1 has been shown to attenuate ischemia/reperfusion damage *in vivo.*[6] Furthermore, HO-1 is an important modulator of atherosclerosis, in part through its ability to direct plaque progression into a more stable phenotype.[7].

The expression and activity of HO-1 is modulated by genetic factors. In the promoter region of the HO-1 gene, located on human chromosome 22q12, two functional polymorphisms have been described: a T(-413)A single nucleotide polymorphism (SNP) and a guanidine thymidine dinucleotide ($(GT)_n$) repeat length or microsatellite polymorphism.[8] The $(GT)_n$ repeat length polymorphism has been studied extensively and long GT repeats have been associated with lower HO-1 activity.[9, 10] In theory, DNA fragments with longer GT repeat lengths have the potential to assume a Z-DNA conformation, which is thermodynamically unfavorable when compared to the B-DNA conformation. This Z-DNA conformation might negatively affect transcriptional activity, by which longer GT repeats thus result in lower HO-1 expression.[11,12] This was confirmed by luciferase promoter constructs and transient transfection assays in different cell lines.[12,13] Altogether, it would indeed only be logical to presume that long GT repeat carriers have an increased risk of cardiovascular disease. Moreover, epidemiological data of the general population confirm that the distribution of the $(GT)_n$ repeat polymorphism differs in patients with cardiovascular disease when compared to patients without cardiovascular disease.[14] More specifically, the bi-allelic long $(GT)_n$ repeat genotype is overrepresented in patients who developed cardiovascular disease.[14] Data on the T(-413)A SNP are rather limited,[15-17] although the A allele is considered to increase the enzymatic activity. Homozygous carriers of the A allele were found to have a decreased risk of ischemic heart disease.[17] The exact mechanism of higher HO-1 activity in the A SNP has not been described however.

Data on HO-1 and atherosclerosis in the context of CKD are sparse to date.[18,19] Recently, in a large cohort of 1080 Asian hemodialysis patients, Chen and colleagues showed an association between the long $(GT)_n$ repeat polymorphism and the risk of

long-term CV events and all-cause mortality.[19] Another study demonstrated that longer $(GT)_n$ in the HO-1 promoter predicted poor arteriovenous fistula (AVF) patency and hence higher incidence of AVF failure in HD patients.[20]
Our aim was to extend the available data from the general population and Asian dialysis patients to Caucasian patients with CKD and study the association between the HO-1 polymorphisms ($(GT)_n$ repeat and T(-413)A SNP) and cardiovascular disease in a dialysis population.

Materials and methods

Patients

205 peritoneal and hemodialysis patients were prospectively included in a single-center study at the University Hospitals Leuven between 2010 and 2013 at admission for transplantation (n=190) or peripheral vascular surgery (n=15).
The study was performed conforming to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospitals Leuven, Belgium. All patients provided written informed consent.

Demographical, clinical and biochemical data

Demographical and clinical data were retrieved from the patient medical files through thorough file review by LJ and KD. Primary renal diagnoses were defined according to the new ERA-EDTA Primary Renal Diagnosis coding system[21] Comorbidity was assessed using the method described by Charlson et al.[22] The cardiovascular risk profile was calculated based on the Framingham Risk factors diabetes, arterial hypertension, smoking (current or quit for less than five years ago), obesity or dyslipidemia, with a maximum score of 5. Dialysis duration was measured as the time in months between start of dialysis treatment and inclusion date.
Arterial hypertension (AHT) was defined as a positive history reported in the medical file and/or current use of antihypertensive drugs. Diabetes was defined as a positive history reported in the medical file and/or current use of glucose lowering treatment. Dyslipidemia was defined as the presence of lipid abnormalities at admission (LDL cholesterol >115 mg/dl or HDL <40 mg/d or triglycerides >150 mg/dl) or current use of lipid lowering medication. Smoking status as well as the use of lipid lowering and anti-hypertensive treatment were derived from the admission files.
The presence of cardiovascular disease was defined based on the results of Doppler/ultrasonography of the carotid and lower limb arteries (atheromatosis on ≥2 vessels), magnetic resonance or computed tomography-based angiography of the lower limbs (≥2 arteries with moderate stenosis), electrocardiography, coronary angiography (≥1-vessel disease or diffuse atheromatosis without significant stenosis), cyclo-

ergometry, echocardiography and/or documented history of acute myocardial infarction (AMI), stable or unstable coronary artery disease, peripheral artery disease, carotid disease with/without intervention.

Blood sampling and genotype analysis

Blood samples were taken on the day of admission.
Hemoglobin (g/dl), C-reactive protein (CRP) (mg/l), creatinine (mg/dl), urea (mg/dl), calcium (mg/dl), albumin (g/l), phosphate (mg/dl), bicarbonate (mmol/L), total cholesterol (mg/dl), HDL (mg/dl), LDL (mg/dl), triglycerides (mg/dl), 25-OH-vitamin D (µg/l)) were measured using standard laboratory techniques. Serum concentrations of full-length (biointact) PTH were determined by an immunoradiometric assay, as described elsewhere.[23] IL-6 was measured in serum using enzyme-linked immunosorbent assays (IL-6 HS ELISA e-Bioscience, Vienna (Austria)), performed according to the manufacturer's instructions.
For genotyping, whole blood was collected in vacutainer EDTA tubes (BD Diagnostics, Plymouth, UK). Genomic DNA was isolated from whole venous blood using a lysing and salting-out procedure as described by Miller et al..[24] In short, buffy coats of nucleated cells obtained from anticoagulated blood (ACD or EDTA) were resuspended in lysis buffer (5 mM $MgCl_2$, 20 mM Tris HCl). The cell lysates were digested overnight at 37°C with 0.5 ml of 10% SDS and 50 µl of a proteinase K solution (20 mg/mL). After complete digestion, cell debris and proteins are removed by adding 2 ml of a 5M NaCl solution. The supernatant containing DNA was transferred to a 15 ml Falcon Tube. DNA precipitation occurred by adding isopropanol. The precipitated DNA strands were removed with a glass hook and transferred to a 1.7 ml microcentrifuge tube containing 500 µL TE buffer. The DNA was incubated overnight at 50°C before quantitating. DNA concentration and spectrometric 260/280 ratios were determined on the Nanodrop 2000c (Thermo Scientific, Breda, Nederland).

1. Determination of the GT-repeat length ($(GT)_n$)

The 5' flanking region of HO-1 was amplified by PCR with one FAM-labeled primer (Veriti® 96-Well 0.1ml Fast Thermal Cycler) using Forward 5'-AGAGCCTGCAGCTTCTCAGA-3' and Reverse 5'-GTCCTATGGCCAGACTTTGT-3'. PCR assays were performed over 25 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C containing in a max volume of 15 µl, 5 µl DNA (10 ng/µl) and 10 µl PCR mix (dNTPs (Roche), Kapa enhancer, Kapa2G Robust (Sopachem). The resulting PCR product was mixed with 0.3 µl Genescan 500 Liz size standard (Applied Biosystems) and 9 µl HiDi formamide, followed by fragment analysis on the ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The GT-repeat length was determined and statistically analyzed using Genescan analyzer (Applied Biosystems). Alleles with <25 GT repeats were defined as short. Genotypes were classified as SS, LS or LL.

2. Determination of the single nucleotide polymorphism T(-413)A (rs2071746)

The SNP mutation was assayed using a TaqMan®SNP Genotyping Assay (Life technologies, Brussels). Briefly, 8 µl mix (Taqman assay 20x, Genotyping mastermix and Baxtrer water) and 2 µl of the gDNA at a concentration of 5 ng/µl were added to the assay plates in the dark, to reach a total working volume of 10 µl. The assay was run on a AB StepOnePlus RT PCR or 7500 fast using the standard run (standard prePCR read, hold 10 min 95°C, 40 cycles denaturing for 15 sec at 92°C, annealing and extension for 1 minute at 60°C, followed by a standard post read phase). Genotypes were classified as AA, AT or TT.

Statistics

Continuous variables are expressed as median and interquartile range. Categorical variables are expressed as percentages. Patient characteristics were compared using nonparametric ANOVA (Wilcoxon Two-Sample test) for continuous data and Fisher's Exact test for categorical data. *P*-values <0.05 were considered significant.

The distribution of the $(GT)_n$ polymorphism and the SNP were tested for consistency with the Hardy-Weinberg Equilibrium. Linkage disequilibrium statistics for the SNP and the $(GT)_n$ polymorphism were visualized by the Haploview 4.2 software. An LD threshold of r2>0.8 was set in the program.

Multivariate logistic regression analysis was performed to define variables independently associated with cardiovascular disease. Three different multivariate models were constructed. In MODEL A all variables found to be univariately associated with the outcome variable on $P<0.2$ were included. The backward elimination technique was used to identify the best subset on $P<0.2$. This subset was then subjected to a final backward selection elimination procedure on $P<0.05$. The initial variable set for MODEL B was composed of the established cardiovascular risk factors and the genotype. MODEL C was constructed by combining the variables introduced in MODELS A and B.

All statistical analyses were conducted with the SAS statistical package, version 9.3.

Results

Patients, demographical, clinical and biochemical data

Table 5.2.1 shows demographical, clinical and biochemical data of all 205 patients included in the analysis. 152 patients were on hemodialysis. 68 percent of the patients were male. Median age was 60 years (51-67). 104 patients with CVD were compared to 101 patients without cardiovascular disease (nonCVD). In the CVD group, 54.8% had documented coronary artery disease, 22.1% had had an AMI, 61.2% had documented

carotid artery disease, 23.1% had had a cerebrovascular accident (CVA) and 34.6% had documented peripheral artery disease. As expected, the proportion of patients with diabetes, dyslipidemia or history of smoking was significantly higher in the CVD group as compared to the nonCVD group. In accordance, their cardiovascular risk profile and Charlson Comorbidity Index were higher. Moreover, they were older, had higher CRP and Interleukin-6 levels and lower serum phosphate. Lower levels of total and LDL cholesterol in the CVD as compared to the nonCVD populations reflect the higher use of lipid lowering therapy in the former.

Table 5.2.1 Demographic and biochemical characteristics of the study populations.

205	nonCVD	CVD	*P*
N	101	104	
N HD / PD	67 / 34	85 / 19	0,001
Male %	66,3	69,2	0,765
Hypertension %	74,3	83,7	0,123
Diabetes %	10,9	42,3	<0,0001
Dyslipidemia %	67	86,5	0,001
Smoking			
Never %	51,5	35,6	0,020
Active or quit < 5 years ago %	23,8	41,3	
Former %	24,7	23,1	
Lipid lowering therapy %	43	76	<0,0001
	Median (IQ range)	Median (IQ range)	p-value
Age	53,0 (41-62)	65,0 (58,5-69,5)	<0,0001
BMI (kg/m²)	24,4 (22,2-27,2)	25,4 (23,2-28,3)	0,101
Number of antihypertensives	1,0 (1-2)	2,0 (1-3)	0,371
Cardiovascular risk score	2 (1-3)	2 (2-3)	0.0001
Charlson index score	3,0 (2-5)	6,0 (5-7)	<0,0001
Dialysis duration (months)	29,0 (16,1-42,9)	35,8 (20,6-46,2)	0,053
Preserved residual renal function %	67	56	0.114
Residual diuresis (ml)	500,0 (20-1000)	225,0 (0-875)	0,123
PTH 1-84 (ng/L)	180,8 (103,9-277,9)	159,0 (83,9-277,8)	0,557
25-OH vitamin D (µg/L)	39,9 (31,1-52,7)	39,2 (29,9-52,2)	0,867
Cholesterol (mg/dL)	171,0 (152-200)	158,0 (132,5-192)	0,009
Triglycerides (mg/dL)	131,0 (103-227)	130,0 (94-221)	0,635
Low-density lipoprotein (mg/dL)	89,0 (69-109)	74,0 (56-94)	0,002
High-density lipoprotein (mg/dL)	48,0 (39-62)	45,0 (36-61)	0,219
CRP (mg/L)	2,9 (1,3-5,7)	4,2 (2-9)	0,015
HCO_3 (mmol/dL)	25,2 (23,4-27,3)	26,0 (23,6-27,8)	0,204
Hemoglobin (g/dL)	12,1 (11,3-12,7)	12,2 (10,9-13,5)	0,507
Albumin (g/L)	44,0 (41,3-46,5)	44,2 (40,5-46,6)	0,911
Phosphate (mg/dL)	4,3 (3,4-5,5)	3,9 (3,3-4,8)	0,033
Calcium (mg/dL)	9,4 (8,9-9,8)	9,4 (8,8-9,8)	0,737
Interleukin 6 (pg/mL)	3,5 (2-5,1)	4,5 (2,5-8,4)	0,010

nonCVD patients without cardiovascular disease; CVD patients with cardiovascular disease; IQ range interquartile range; BMI body mass index; PTH 1-84 parathyroid hormone 1-84; CRP C-reactive protein; HCO_3 bicarbonate.

HO-1 promoter polymorphisms

Both the genotype distribution of the $(GT)_n$ polymorphism and the T(-413)A SNP fulfilled the Hardy-Weinberg equilibrium (HWE *P*=0.3189 and HWE *P*=0.0654, respectively).

Figure 5.2.1 shows the allelic distribution of the $(GT)_n$ repeat polymorphism in the study population. The distribution was bimodal with 23 and 30 repeats as the most common repeat lengths, which is consistent with the literature.[10]. Overall, in the studied dialysis population, the $(GT)_n$ genotype distribution classified as bi-allelic short (SS), bi-allelic long (LL) and heterozygous 'short' and 'long' (SL) was 8.6%, 55% and 36%, respectively. Table 5.2.2a shows the genotype distribution for CVD and nonCVD populations separately. The proportion of the SS-genotype was significantly lower in the CVD compared to the nonCVD population (4.9% vs. 12%). The OR for CVD in the LL vs. SS-genotype was 3.148 [1.039-9.541] (*P*=0.043).

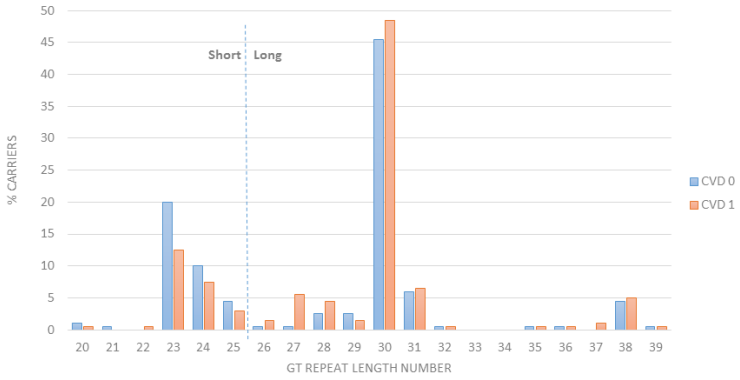

Figure 5.2.1 *$(GT)_n$ allelic distribution for CVD (n=101) vs nonCVD (n=100).* The frequency of each number of GT repeats is given in % of the total CVD (red) and nonCVD (blue) patients.

Distribution of the T(-413)A SNP was: AA 39.2%; AT 39.2%; TT 21.6%. There was no significant association between the SNP and CVD in our study population.

Twenty-seven percent of the patients had the combination of at least one short $(GT)_n$ repeat allele and at least one A SNP allele. This combination of genotypes can theoretically be considered as having a higher HO-1 activity as compared to the remainder of the patients. However, with 43.6% and 52.7% of CVD, there were no significant associations with presence or absence of CVD in these genotype combination groups (Table 5.2.2b). In another approach, patients with at least 2 favorable alleles (i.e. short GT-repeat and/or A variant of the SNP) were compared

with patients with 1 or no favorable allele (Table 5.2.2c). Again, there were no significant associations with presence of CVD (48.9% vs. 55.8%).

Table 5.2.2a Genotypical data of all included patients.

GT repeat length*	%SS	%SL	%LL	OR LL vs. SS
CVD	4,9%	32,7%	62,4%	3,148 [1,039-9,541]
nonCVD	12%	40%	48%	*P*=0.043
T(-413)A SNP**	%AA	%AT	%TT	OR TT vs. AA
CVD	41,4%	39,4%	19,2%	0,714 [0,335-1,506]
nonCVD	37%	39%	24%	*P*=0.378

* GT repeat genotyping data were available in 101 and 100 patients in the CVD and nonCVD groups, respectively. ** SNP genotyping data were available in 99 and 100 patients in the CVD and nonCVD groups, respectively. OR, odds ratio.

Table 5.2.2b Comparison of patient subgroups with at least one copy of each favorable allele versus others.

	nonCVD patients	CVD patients	*P*-value
% with ≥1 A allele and ≥1 S allele*	56,4%	43,6%	0.271
% others**	47,3%	52,7%	

* 55 patients had at least one A allele and at least 1 short GT repeat length allele; ** 146 patients did not have both an A allele and a short GT repeat length allele

Table 5.2.2c Comparison of patient subgroups with at least two favorable alleles versus others.

	nonCVD patients	CVD patients	*P*-value
% with 2-3 favorable alleles*	51,1%	48,9%	0.421
% with 0-1 favorable alleles**	44,2%	55,8%	

* 139 patients had at least 2 favorable alleles (SNP and/or GT repeat length); **52 patients had only 1 or no favorable alleles (SNP and/or GT repeat length)

Multivariate logistic regression analysis for CVD

As explained in the methodology section, three different multivariate models were constructed. While model B contains only the established cardiovascular risk factors (age, gender, diabetes, arterial hypertension, smoking and dyslipidemia) and the $(GT)_n$ genotype, model A and C are constructed using all variables that show univariate association with CVD in the comparative analysis and/or logistic regression analysis. Model C, which combines variables introduced in Models A and B, differs from model A only by the addition of the gender variable. The models are depicted in Table 5.2.3. Age and diabetes are consistently retained as independent factors associated with cardiovascular disease (CVD) and dialysis duration is an additional independent variable in Models A and C. GT repeat length, which was shown to be univariately associated with CVD (Table 5.2.2), lost significance in the multivariate backward elimination models.

Table 5.2.3 Multivariate logistic regression analysis.

MODEL A

Variables introduced in the model*
AHT, diabetes, dyslipidemia, smoking, age, BMI, dialysis duration, residual diuresis, CRP, phosphate, IL-6, GT repeat length
Selected subset of variables after initial screening:
Age, diabetes, dialysis duration, phosphate, dyslipidemia
Variables consecutively removed from the model by backward elimination (p-value at elimination):
Dyslipidemia (0.3331), phosphate (0.0725)
Final model:

Variable	OR (95% CI)	*P*-value
Age (unit = 5 years)	1.645 (1.370-1.975)	<0.0001
Diabetes	5.305 (2.260-12.452)	0.0001
Dialysis duration	1.092 (1.001-1.191)	0.0481

MODEL B

Variables introduced in the model:
AHT, age, diabetes, dyslipidemia, gender, smoking, GT repeat length
Selected subset of variables after initial screening:
Age, diabetes, GT repeat length
Variables consecutively removed from the model by backward elimination (p-value at elimination):
GT repeat (0.0863)
Final model:

Variable	OR (95% CI)	*P*-value
Age (unit = 5 years)	1.628 (1.368-1.937)	<0.0001
Diabetes	4.411 (1.997-9.740)	0.0002

MODEL C

Variables introduced in the model*:
AHT, diabetes, dyslipidemia, smoking, age, BMI, dialysis duration, residual diuresis, CRP, phosphate, IL-6, GT repeat length, gender
Selected subset of variables after initial screening:
Age, diabetes, dialysis duration, phosphate, dyslipidemia
Variables consecutively removed from the model by backward elimination (p-value at elimination):
Dyslipidemia (0.3331), phosphate (0.0725)
Final model:

Variable	OR (95% CI)	*P*-value
Age (unit = 5 years)	1.645 (1.370-1.975)	<0.0001
Diabetes	5.305 (2.260-12.452)	0.0001
Dialysis duration	1.092 (1.001-1.191)	0.048

*Cardiovascular risk score, Charlson comorbidity score, total cholesterol, LDL cholesterol and use of lipid lowering therapy were not introduced in the model, given their obvious correlation with individual variables such as hypertension, diabetes and dysplipidemia. AHT arterial hypertension; BMI body mass index; CRP C-reactive protein; IL-6 interleukin 6

Association of age and diabetes with the $(GT)_n$ HO-1 promoter polymorphism

Neither diabetes nor age (above or below median age) showed significant associations with the GT repeat polymorphism distribution in our population (Tables 5.2.4 and 5.2.5). Accordingly, logistic regression analysis for CVD of LL vs. SS genotypes showed no significant odds ratios in subgroups of patients with diabetes, age above or below median age (data not shown). Subgroup analysis of patients without diabetes, however, remained the univariate association between long $(GT)_n$ repeat length polymorphism and CVD (OR 9,721 [1.199-78.834], *P*=0.03).

Table 5.2.4 Association between $(GT)_n$ repeat polymorphism and diabetes.

GT repeat length	%SS	%SL	%LL	OR LL vs. SS
DM	9.2	35.2	55.5	0.889
no DM	8.2	36.7	55.1	(0.289-2.736)
				P=0.837

OR, odds ratio; DM, diabetes mellitus

Table 5.2.5 association between $(GT)_n$ repeat polymorphism and age (median age = 60 years).

GT repeat length	%SS	%SL	%LL	OR LL vs. SS
Age above median (≥60)	4.9	35.3	59.8	2.926
Age below median (<60)	12.1	37.4	50.5	(0.966-8.863)
				P=0.058

OR, odds ratio

Discussion

Our study, a cross-sectional cohort study of 205 dialysis-dependent patients, is the first to show that the association between $(GT)_n$ repeat length polymorphism of the HO-1 gene and CVD seen in the general population also applies to a Caucasian population of patients with advanced CKD. This finding reinforces the report of Chen and colleagues, who recently demonstrated the association of the LL-genotype and the risk of future cardiovascular events in a 50 months follow-up study of 1080 Asian hemodialysis patients.[19]

As Chen and colleagues mentioned, their findings are applicable to the Taiwanese population only and corroborating validation studies in other populations are needed. This is of particular relevance considering the difference in $(GT)_n$ repeat genotype distribution between Caucasians and Asians, as concluded from a recent meta-analysis of the available literature.[14] Indeed, the ethnic profile of a cohort should not be overlooked because racial or ethnic background can be a key factor in genotype/phenotype associations.

Although it might seem evident that a variable associated with CVD in the general population would automatically translate into an association with CVD in a dialysis population, this is not necessarily true. Traditional risk factors such as diabetes, smoking and age are indeed associated with CV outcomes in both dialysis and general populations. But obesity, a well-known risk factor for worse outcomes in the general population, protects people from dying once they have reached end-stage renal disease.[25] Several explanations have been given for this phenomenon of "reverse epidemiology". Amongst them is the notion that dialysis patients constitute a highly selected population,[26] to which counter-intuitive associations may apply. The distribution of the HO-1 $(GT)_n$ genotypes in the studied dialysis population (SS 8.5%, LL 55% and SL 36%) is different from what is seen in a general Caucasian population[14] (SS 11%, LL 46% and SL 43%, *P*=0.034). This supports the idea that dialysis patients constitute a selection of the population with a relatively higher percentage of the low HO-1 expressing genotype. Nevertheless, even within this highly selected group, our findings confirm the association of the gene's promoter polymorphism with CVD (Table 5.2.2a).

After multivariate logistic regression analysis, however, this effect loses significance in favor of other risk factors. The multivariate analysis (Table 5.2.3) shows that the presence of diabetes, higher age and longer dialysis duration are independently associated with CVD in our study population. The first two are well-established risk factors for CVD. However, it is worth questioning whether diabetes and age modify rather than abrogate the effect of the HO-1 $(GT)_n$ polymorphism on CVD. This is of particular interest for diabetes, since the risk of developing type 2 diabetes (T2DM) was found to be associated with long $(GT)_n$ repeats.[9,27] As an example Song et al, in a Chinese case-control study, found that a cohort of newly diagnosed T2DM patients carried a significantly higher percentage of the LL genotype compared to their non-diabetic controls.[9] Such an interaction between the LL genotype and T2DM would theoretically modify the effect of $(GT)_n$ repeat polymorphism on CVD in our population. However, we did not find any relationship between the $(GT)_n$ repeat polymorphism and diabetes mellitus (Table 5.2.4) or age (Table 5.2.5) in our cohort. Within the subgroup of non-diabetic patients (n=150), the univariate association between the $(GT)_n$ repeat length polymorphism and CVD was confirmed, while it was not seen in the subgroup of diabetic patients (n=55) nor in two subgroups based on median age (median age=60 years old). We feel, however, that no firm conclusions can be drawn from these subanalyses, given the low numbers and the consequent presumable lack of power.

Our results show no significant association between the T(-413)A SNP and CVD (Table 5.2.2a). Although it cannot be excluded that an insufficient patient number explains the lack of an association in our population, several other explanations are more likely. First, it is of note that only a limited number of papers point to associations between the SNP and clinically relevant outcomes. This contrasts with the numerous reports on clinical associations with the $(GT)_n$ repeat. This observation at least suggests that the

lack of an association with the SNP is seen more often than reported and that publication bias may explain the low number of reports on this topic.

Second, a linkage disequilibrium (LD) between $(GT)_n$ repeat and SNP genotype has been described by other groups.[9,16,28] We found a moderate LD between the SNP and the $(GT)_n$ repeat, with a D' 0.89 and r^2 0.427. In other words, patients with an unfavorable $(GT)_n$ repeat genotype are likely to have a favorable SNP and *vice versa*. This is not surprising from an evolutionary viewpoint, but it obviously complicates the interpretation of genotype-phenotype associations. From this perspective, our findings suggest that the $(GT)_n$ repeat genotype is probably more important for the cardiovascular phenotype than the SNP. In a mechanistic study, Song and colleagues examined the association of both the T(-413)A SNP and the $(GT)_n$ repeat polymorphism with T2DM.[9] They evaluated HO-1 expression in peripheral blood mononuclear cells according to haplotype combinations. In accordance with what is suggested by our clinical association data, their report clearly shows a greater impact of the $(GT)_n$ repeat polymorphism SS vs. LL than the T(-413)A SNP AA vs TT on HO-1 activity.

Several limitations of our study need to be addressed. First, allocation of patients to the CVD and nonCVD groups was based on retrospective file review. The review process was rigorously performed by two investigators who were blinded for the genotype. All available file data and investigations for documentation of CVD were taken into account as mentioned in the methods section. Moreover, although the analysis was retrospective, all patients had been thoroughly screened according to a standardized preoperative protocol with particular focus on the cardiovascular status. Residual bias can, however, not be excluded completely. A second limitation of our observation may be related to the power of our study. In particular, subgroup analyses suffer from lack of sufficient power. The findings of Chen and colleagues in 1080 HD patients, however, suggest that higher patient numbers would only have strengthened our findings. A third issue worth mentioning is the fact that no direct biological endpoint of enzymatic HO-1 activity was measured in our patients. While it is generally accepted and supported by luciferase promoter constructs and transient transfection assays in different cell lines that the studied polymorphisms affect HO-1 enzymatic activity, a biological in vivo assay of enzymatic activity would have strengthened our results. Finally, the studied population is a selected subgroup of CKD5D patients who are younger and have lower comorbidity scores than the average patients in our and many other Western European dialysis centers. Further investigation is warranted to explore the associations in predialysis CKD patients as well as in non-selected CKD5D patients.

In conclusion, our study provides evidence for a role of the $(GT)_n$ repeat promoter polymorphism of the HO-1 enzyme in the occurrence of CVD in a dialysis population. Dialysis patients with the LL genotype (lower HO-1 expression) have an increased risk for CVD compared to patients with the SS genotype (higher HO-1 expression). Further mechanistic exploration of the role of the HO-1 enzyme in CVD in CKD is needed.

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CHAPTER 6

General discussion

General discussion

In advanced kidney disease the prevalence of atherosclerotic cardiovascular disease is high and nearly equivalent across age groups. Recent data indicate that the impact of renal insufficiency on cardiovascular disease already begins with minor renal dysfunction.[1] Many authors hypothesize that increased oxidative stress is the unifying concept that initiates and feeds this problem.[2]

The pro-oxidative state of chronic kidney disease (CKD)

Several findings in uremic patients point to an imbalance favoring the pro-oxidative state: uremia-related as well as dialysis-related factors (**Table 1.2** of **Chapter 1**) have been described to lead to the increase of oxidants and decrease of the antioxidative capacity.[3] Summarizing atherosclerosis mechanisms in the particular context of CKD (**Chapter 1**), we identified literature evidence on uremia-related increased activity of oxidative enzymes such as NADPH oxidase, myeloperoxidase and xanthine oxidase. Furthermore, some enzyme systems appear to be dysregulated in uremia, such as in the phenomenon of eNOS uncoupling. Also, there is evidence for the accumulation of secondary radicals and transition metals. Additionally, many crucial antioxidative mechanisms have been found to be impaired in CKD patients. A promising field of renewed interest constitutes interventions for simple dietary deficiencies and newly discovered supplements such as flavonols and tempol. Still, many oxidant and certainly antioxidative systems remain relatively unexplored in the context of CKD.

It is clear that the pro/anti-oxidative balance in uremia is disturbed on many levels and that the resulting increase in oxidative stress is multifactorial and self-sustaining. From this it can be easily understood that repairing only one mechanism will unlikely result in clear clinical benefits. This is supported by clinical trials of antioxidant supplementation in atherosclerosis that failed to show substantial benefit in ESRD populations.[4,5] Indeed, a plausible explanation for the negative trial results is that the used antioxidant supplementation is directed against free radicals (superoxide, peroxynitrite), whereas non-radical oxidants such as hydrogen peroxide remain largely unaffected.[6] On the other hand, when enzymatic system disturbances lead to the depletion of antioxidants, supplementation will not be sufficient to restore the enzymatic imbalance. In the same line of thought, absence of a functional NADPH oxidase inhibited $^{\cdot}O_2^{-}$ generation in the aorta of ApoE^{-}/$^{-}$ mice, but did not affect the progression of atherosclerosis.[7,8] However, over-expression of both catalase and SOD was able to markedly inhibit the atherosclerotic lesion progression.[9] Also, statins, the most effective agents for atherosclerosis treatment so far, exert many antioxidative effects by both inhibiting oxidative and inducing anti-oxidative enzymes.[10,11] In CKD and particularly dialysis patients however, statin therapy and LDL lowering was shown not to be as effective as

in the general population.[12-14] The goal of interventional strategies will thus be to target those processes that reach the broadest possible range of involved pathways and change both enzymatic and nonenzymatic oxidative dysregulation. In this respect stress-responsive antioxidative mechanisms such as the ARE-driven enzymes are of interest. These enzymes can be 'switched on' in times of increased oxidative stress. They do not only provide direct antioxidants, but also inactivate oxidants, increase levels of glutathione synthesis and regeneration and stimulate NADPH.

Heme oxygenase-1

The present work focused on one of these central ARE driven anti-oxidant defense mechanisms, notably heme oxygenase-1 (HO-1). This enzyme can be induced by a variety of oxidative agents. It catalyzes the oxidative detoxification of excess heme resulting in equimolar amounts of free iron (Fe^{2+}), biliverdin and carbon monoxide (CO). All products formed in this process are potentially beneficial, since they mediate anti-inflammatory, anti-oxidant and anti-apoptotic effects.[15] HO-1 is involved in the atherosclerotic process, demonstrated by the fact that immunostaining shows its presence in macrophages, foam cells, endothelial cells and vascular smooth muscle cells of atherosclerotic arterial sections. Moreover, in HO-$1^{-/-}$ ApoE$^{-/-}$mice accelerated and more advanced atherosclerotic lesion formation was seen as compared to HO-$1^{+/+}$ ApoE$^{-/-}$ mice, illustrating the protective properties of the enzyme.[16,17] The expression of the human HO-1 gene, located at chromosome 22q12, is modulated by two functional polymorphisms in the promoter: a $(GT)_n$ repeat length polymorphism and a T(-413)A single nucleotide polymorphism (SNP).[18] HO-1 genotype polymorphisms have been demonstrated to play a role in acute kidney injury, chronic inflammatory diseases and in solid organ transplantation. Many clinical observations also show that genotypes with higher HO-1 activity offer protection against atherosclerotic disease, although the literature is not unequivocal in this regard. One of the problems with the interpretation of the literature data is the great diversity in study populations (race, co-morbidity…), genotyping methodology and end-point definitions.

HO-1 promoter polymorphisms and CVD

We performed a systematic review of the epidemiological literature between 1997 and 2013 on the HO-1 (GT)n repeat polymorphism and cardiovascular disease (**Chapter 5.1**). High-quality information was selected based on strict methodological criteria and homogeneous subpopulations were defined. The analysis showed that the short repeat SS-genotype is represented in a lower proportion of patients with established cardiovascular disease as compared to a population without cardiovascular disease or

control patients of whom the cardiovascular status was not explicitly assessed. Consequently, the odds for cardiovascular disease was higher in patients carrying the long repeat LL-genotype as compared to their heterozygous or homozygous short allele carriers. The second but probably even more relevant finding of this review was that racial disparities in HO-1 (GT)n repeat length distribution exist. In the pooled Caucasian populations we found an overall proportion of the SS-genotype of 11%, whereas in the Asian population this reaches up to 22% (see **Table 5.1.2b-c**, **Chapter 5.1**). The difference is even more pronounced when considering S-carriers (SS and SL) (see **supplemental Table 5.1.3b-c**, **Chapter 5.1**) This finding may not be surprising, since it is in agreement with evolutionary theories. Indeed, genetic clusters have arisen due to geographical, social, and cultural barriers isolating human populations. Previous research has shown that allele variants with frequencies of ≥20% tend to occur in multiple racial groups. Variants with frequencies <20% are more likely to be race specific.[19] The racial profile of a cohort should therefore never be overlooked when studying genotype/phenotype associations. Pooling of data from two populations with different racial genotype distributions may indeed be problematic. In our study, given the unequal representation of CVD and nonCVD patients in the Asian and Caucasian subpopulations, the epidemiological evidence supporting the anti-atherogenic effect of the HO-1 polymorphism is clearly overinterpreted in the pooled analysis (**Table 5.1.2a** and **online Table S5.1.3a**, **Chapter 5.1**). Nevertheless, even after stratifying for ethnicity, LL carriers have increased odds ratios for CVD (see **Tables 5.1.2b-c** and **supplemental Tables S5.1.3b-c**, **Chapter 5.1**).

HO-1 promoter polymorphisms and CVD in CKD

Given the protective effect of the (GT)n repeat polymorphism on cardiovascular disease development in the general population, we studied whether this could be confirmed in patients with advanced CKD (**Chapter 5.2**). In a cross-sectional cohort of 205 dialysis-dependent patients, the association between the (GT)n repeat promoter polymorphism and prevalent CVD was indeed confirmed. No effect was seen however of the T(-413)A SNP, which was studied simultaneously in the same study population. We feel that the latter finding is not unexpected. First, it is of note that there is only a limited number of papers pointing to associations between the SNP and clinically relevant outcomes. This contrasts with the numerous reports on clinical associations with the (GT)n repeat. This observation at least suggests that the lack of an association with the SNP is seen more often than reported and that publication bias may explain the low number of reports on this topic. Second, a linkage disequilibrium between (GT)n repeat and SNP genotype has been described by other groups.[20,21] We found a moderate LD between the SNP and the $(GT)_n$ repeat, with a D' 0.89 and r^2 0.427. In other words, patients with an unfavorable (GT)n repeat genotype are likely to have a favorable SNP and vice versa.

This is not surprising from an evolutionary viewpoint, but it obviously complicates the interpretation of genotype-phenotype associations. From this perspective, our findings suggest that the (GT)n repeat genotype is probably more important for the cardiovascular phenotype than the SNP. Having said this, the positive association between the (GT)n repeat polymorphism and CVD was only found in univariate analysis. The findings suggest that the protective effect of HO-1 is easily overruled by the known traditional cardiovascular risk factors (diabetes and age) as well as dialysis duration. Although not directly evident from our analysis, one might argue that the latter variable represents at least some of the many non-traditional uremia-related risk factors mentioned earlier (**Chapter 1**).

HO-1 and atherosclerosis in CKD

While the genotype study allowed us to confirm the protective role of the short (GT)n repeat promoter polymorphism in patients with ESRD, another objective of our project was to study how uremia affects the expression and activity of HO-1 in atherosclerosis and inflammation. One might expect HO-1 transcription and activity to be stimulated in chronic kidney disease (CKD) as a consequence of the overall pro-oxidant and pro-inflammatory state. Alternatively, however, the uremic milieu – as it is the case for other enzymes (e.g. dimethylarginine demethylaminohydrolase (DDAH)) – may interfere with normal HO-1 function and abrogate its anti-oxidant potential.
Findings in kidney injury models are in favor of the latter hypothesis. Kim et al. reported impairment of the Nrf2-Keap1 pathway and its target genes, amongst others HO-1, after six and twelve weeks of CKD in a rat remnant kidney model, despite clear evidence of increased oxidative stress and inflammation.[22] In addition, reversing the HO-1 deficiency in the failing kidney by hemin induced activation of the enzyme led to an attenuation of chronic tubulo-interstitial kidney damage.

To study the expression of HO-1 in the context of atherosclerosis and CKD, we evaluated atherosclerotic plaque morphology and HO-1 expression in patients with known advanced atherosclerotic disease of the peripheral arterial vasculature (**Chapter 3**). In 66 biopsies of arteries procured during clinically indicated vascular surgery procedures, atherosclerotic lesions of CKD patients were characterized by higher inflammatory cell infiltration, higher MCP1 and Caspase-3 expression, more neovascularization and a higher proportion of plaque complications than those of nonCKD patients. Moreover, eGFR was found to be an important predictor of both intra-plaque neovascularization and atherosclerotic plaque complications, independent of traditional cardiovascular risk factors. Interestingly, by higher inflammation and neovascularization, even the morphologically organized lesions of CKD patients retain a "remodeling phenotype", as illustrated in **Figure 7** of **Chapter 3**. These findings all fit

within a 'vulnerable plaque'-phenotype as previously defined by several authors.[23-26] Indeed, thin fibrous caps, high infiltration by (foamy) macrophages, a lipid-rich necrotic core and high levels of neovascularization are characteristics of plaques with high risk for rupture or thrombosis.

Immunohistochemical HO-1 intensity was not significantly different between the nonCKD and CKD groups. The mRNA expression analysis, being more sensitive and accurate than semi-quantitative immunohistochemical scoring systems, even showed a significantly lower proportion of *HO-1* mRNA upregulation in the CKD as compared to the nonCKD group.

In line with the stress-responsive nature of HO-1, studies in non-uremic atherosclerosis such as the one by Cheng and colleagues, showed that HO-1 expression was upregulated in human vulnerable plaques.[16] Moreover, HO-1 correlated positively with local matrix metalloproteinases, various cytokines such as IL-6 and IL-8 and percentages of plaque lipids and macrophages.

As such, our findings in CKD-related atherosclerosis suggest that in a uremic environment there is relatively insufficient activity of HO-1, and that this may contribute to the higher level of inflammation, neovascularization and plaque complications seen in the atherosclerotic plaques of CKD patients. It is of note that correlation analysis on the entire cohort (comprising CKD and non-CKD patients together) showed that immunohistochemical HO-1 expression is positively associated with inflammation and neovascularization. These correlations are maintained in a subanalysis of the nonCKD patients only, but seem to be lost in the subgroup of CKD patients (data not shown). While the subgroup analyses may obviously be hampered by power issues, the lack of an association between HO-1 expression and these inflammatory markers may as well again suggest relative deficiency of the HO-1 defense mechanism in uremia.

We must acknowledge that lesion maturity is of potential influence on HO-1 expression. Again, taking into account the stress-responsive nature of HO-1, the enzyme's activity could be transiently increased only in acute phases of the atherosclerotic process rather than being expressed constitutively throughout the entire lifetime of atherosclerotic plaques. In our study subjects, lesions were of different 'ages', but each of them had evolved beyond the initiating event of atherosclerosis at time of biopsy. This evidently could have confounded associations between HO-1 expression and vulnerable plaque characteristics. Study of early atherosclerotic plaques would be helpful to further explore the associations. From the viewpoint of feasibility and ethical acceptability however, a human study of early (and obviously clinically asymptomatic) atherosclerotic biopsies would be very hard to accomplish.

HO-1 expression in response to oxidative stress in uremic conditions

To further explore the effect of the uremic environment on HO-1 expression in the early phase of atherogenesis, we conducted an *in vitro* study using human umbilical arterial endothelial cells preconditioned in either healthy or uremic human serum conditions (**Chapter 4**). Endothelial dysfunction associated formation of peroxynitrite ($ONOO^-$) plays an important role in the initiation of atherogenesis and protein nitration, "the footprint of $ONOO^-$ generation", has been found in atherosclerotic lesions.[27,28] Therefore, we exposed endothelial cells to SIN-1 which, during its decomposition in the culture medium, slowly releases $ONOO^-$ and as a consequence mimics the early atherogenic oxidative stress.

Using this approach, we found an increased vulnerability of the cells in the uremic conditions in response to oxidative stress, which is in line with the finding of the "vulnerable plaque phenotype" in the clinical study (**Chapter 3**). Indeed, when exposed to increasing concentrations of the short-lived oxidative stressor $ONOO^-$ and to SIN-1 at 7.5 mM, cell toxicity and apoptosis occur at lower concentrations of the oxidants and to a larger extent in uremic conditions. In other words, when exposed to equal amounts of oxidative stress, healthy preconditioned endothelial cells seem to react adequately by upregulating defense mechanisms, whereas uremic preconditioned cells activate apoptotic pathways and cell integrity is lost. HO-1 expression, the defense mechanism of interest in this project, however, was not different between healthy and uremic conditions when measured in these cells. Except for slightly higher baseline mROS measurements in uremic vs. healthy conditions, ROS measurements were not impacted by the uremic state per se. This may seem somewhat counterintuitive given the existing data on uremia as a pro-oxidative state as described in detail in Chapter 1.
Directly measured cROS and mROS are obviously just a small piece of the puzzle of the human oxidant/antioxidant mechanisms. A proteomics study by Carbó et al found indeed upregulated antioxidant genes such as superoxide dismutase (SOD) and gluthathione peroxidase (GPx) after the exposure of human umbilical venous endothelial cells (HUVECs) to uremic serum.[29] At the same time however, decreased SOD and GPx activities in uremia have been described.[30,31] Also, deficiency of ROS scavengers such as ascorbic acid, tocopherol, bilirubin and albumin have been described in CKD.[32,33] The measured cytoplasmic ROS in our experiments is considered mainly the result of the release of $ONOO^-$ by the degradation of SIN-1. Mitochondrial ROS result from the mitochondrial respiration and electron leakage and are implicated in aging as well as in a range of degenerative diseases.[34] The effect of the generated cROS and changes in mROS on the generation of oxidation end-products such as nitrotyrosine, DNA damage or on the generation of non-radical oxidizing molecules participating a.o. in lipid oxidation, however, was not quantified in the present study.

Off course, next to other uremic features, systemic inflammation will most likely be an accomplice in the increased 'uremic' vulnerability to oxidative stress. This is evident from the higher inflammatory cell infiltration in the atherosclerotic plaques of CKD patients, described in Chapter 3. But we also found higher CRP levels in the uremic serum pool used in the experiments in Chapter 4. However, preconditioning with US, containing more (low-grade) inflammation than HS, did not result in a higher HO-1 expression in the uremic cells. This could again be interpreted as a relative failure of antioxidative defense mechanisms in uremia, with subsequent oxidative damage.

HO-1 induction and overexpression

Notwithstanding this interpretation, we found that an interventional strategy, consisting of strong induction of HO-1 was capable of abrogating most of the apoptotic manifestations, including the occurrence of cellular oxidative stress. Hemin is a strong and selective inducer of HO-1.[35] Circulating serum concentrations under treatment reach up to 100 µM. Hemin pretreatment was able to induce a strong HO-1 expression in both healthy and uremic conditions without compromising the cells oxidative metabolism or vulnerability (**Chapter 4**). Moreover, this hemin-induced overexpression of HO-1 led to the protection of cells against the imposed oxidative stress in both the healthy and uremic conditions. These findings clearly confirm the relation between cellular integrity and pro-oxidative imbalance in uremia, an imbalance that can be restored by raising the cytoplasmic HO-1 concentrations to artificially high levels. Our findings uncover an interesting possibility for potential therapeutic interventions. Indeed, as Hemin is available as a registered drug for the treatment of attacks of acute intermittent porphyria, its use for potent HO-1 induction in other medical indications would broaden its field of applicability. In a recent phase IIb study Hemin preconditioning was evaluated in deceased donor renal transplantation for its potential to reduce ischemia-reperfusion related injury[36] and it was demonstrated for the first time that Hemin safely induces HO-1 in transplant recipients. Of course, it remains to be further explored whether and by which regimen such a treatment could be useful in a more chronic process like atherogenesis. Also, the exact mechanism by which HO-1 overexpression offers protection in our model of arterial endothelial cells needs further elucidation. Several mechanisms could be hypothesized, based on the anti-oxidative and anti-inflammatory role of the end-products of HO-1 enzyme activity (**Figure 1.2**, **Chapter 1**).

First, the release of bilirubin provides an increase in the antioxidative defense capacity. Bilirubin itself functions as ROS scavenger. Additionally both free and albumin-bound bilirubin are able to reduce α-TO$^{\cdot}$ to α-TOH and to inhibit LDL lipid peroxidation.[37] Second, carbon monoxide has been shown to act in a similar way as nitrogen oxide, by activating soluble guanylyl cyclase (sGC) and induce cGMP formation[38]. Since

endothelial dysfunction is actively involved in the initiation of the atherosclerotic process and eNOS uncoupling is a well-known phenomenon in uremia,[39] one could argue that the availability of a molecule with similar action as NO could be of beneficial value. Further studies confirmed CO's important biological activities: vasomotor relaxation, antiplatelet aggregation, and antiapoptosis of endothelial cells.[40] Moreover, HO-1 influences many inflammatory pathways, again mainly mediated by CO: pro-inflammatory cytokine production of TNFα, IL-1, IL-6, MCP-1 is inhibited and the anti-inflammatory cytokine IL-10 is induced.[41] Third, the released free iron by HO-1 is considered to rapidly induce the formation of ferritin and an ATPase pump that actively removes intracellular iron from the cell. The resulting modulation of the intracellular iron store and increased iron efflux are believed to be another mechanism by which HO-1 confers resistance to oxidative stress.

Next to the exploration of the exact mechanism by which HO-1 induction offers protection in CKD-related atherogenesis, some other questions need further evaluation. First, one might ask whether the extent of the protective effect of HO-1 overexpression varies with different genotypes of the promoter of the gene. As shown in **Chapter 5.1** and **5.2**, HO-1 promoter polymorphisms are associated with the prevalence of CVD in general as well as in CKD populations. However, neither the atherosclerotic lesions in **Chapter 3**, nor the HUAECs in **Chapter 4** were stratified according to their HO-1 genotype and as a consequence no differential HO-1 expression effects were studied. A second point of interest concerns the effect of HO-1 overexpression in mononuclear cells and vascular smooth muscle cells in CKD vs. non-CKD conditions. Within the framework of the present project, only endothelial cells were studied. The study of human peripheral mononuclear cells, isolated from the blood of dialysis patients and healthy controls would be of particular interest, since these cells would reflect a true chronic uremic and healthy status, respectively.

As already mentioned, recently in a phase IIb study, Hemin preconditioning was evaluated in deceased donor renal transplantation for its potential to reduce ischemia-reperfusion related injury.[36]

Another recent trial, the BEACON trial, studied bardoxolone methyl, a Nrf2-inducing agent. As a consequence of its mode of action, bardoxolone methyl induces HO-1 amongst many other Nrf2-driven enzymes.[42] The BEACON trial was a phase 3, randomized, double-blind, parallel-group, international, multi-center trial of once-daily administration of bardoxolone methyl in patients with type 2 diabetes mellitus and stage 4 CKD who were also receiving guideline-based conventional therapy. The trial design was based upon the findings of the BEAM trial in 227 patients with type 2 diabetes mellitus and CKD (eGFR between 20 and 45 ml/min/1.73 m^2), which had shown that bardoxolone methyl reduces inflammation and oxidative stress[43] and induces an increase of the estimated GFR. The BEACON trial, however, was terminated early because of safety concerns, driven primarily by an increase in cardiovascular events in the bardoxolone methyl group,[44] notably heart failure, nonfatal myocardial

infarction, nonfatal stroke and death from cardiovascular causes. The exact mechanism linking bardoxolone methyl to these cardiovascular events remains unknown. However, the authors suggested that an increase in preload due to volume expansion and an increase in afterload (as reflected by increased blood pressure), coupled with an increase in heart rate, constitute a potentially potent combination of factors that are likely to precipitate heart failure in an at-risk population. Still, it seems counterintuitive to find cardiovascular events resulting from a HO-1 inducing agent, when HO-1 has so often been shown to be athero-protective. However, it must be acknowledged that Bardoxolone Methyl is an inducer of Nrf2, a transcription factor that leads to the induction of many pathways and enzymes other than HO-1. Thus pro-atherogenic pathways may be induced as well. Indeed, Nrf2, in addition to coordinating cellular defenses against electrophilic agents and ROS, also plays an essential role in the regulation of CD36 expression.[45] Plenty of evidence exists pointing towards a pro-atherogenic effect of Nrf2 through the induction of CD36.[46] CD36 is a pleomorphic molecule which functions as a B-scavenger receptor for oxidized low-density lipoprotein (oxLDL) and the interaction with oxLDL triggers signaling cascades for inflammatory responses[47] rendering CD36 mainly a pro-atherogenic molecule.[48,49] Another explanation for the cardiovascular events resulting from bardoxolone methyl has been provided by Van Laecke et al. They consider the well-known side-effect of hypomagnesemia and its association with the risk to develop heart failure with preserved ejection fraction as a potential culprit.[50] From this background, we do not feel that the disappointing results of the BEACON trial should prompt the research community to stop investigating the options for HO-1 induction with more selective approaches, such as Hemin.

General methodological remarks

Probably one of the most important limitations of our studies is the lack of quantification of reliable and reproducible oxidation end products. In the *in vitro* study (**Chapter 4**) only cROS and mROS were determined. It is however unclear whether oxidation end-products are directly proportional to the amounts of the detectable ROS and whether this is different between healthy and uremic conditions. $ONOO^-$ typically leads to the generation of nitrotyrosine[51]. Protein nitration is considered the 'footprint' of $ONOO^-$ generation, but also lipids, DNA and enzymes could be oxidatively modified by $ONOO^-$. The measurement of additional oxidation end products could have resulted in further insights in the mechanism of the higher vulnerability in uremia. Likewise, in the clinical study on atherosclerotic biopsies (**Chapter 3**), F2-Isoprostanes were determined in all included patients as a stable representation of lipid peroxidation, but did not show any difference between nonCKD and CKD patients. It should be noted that all patients included in the latter analysis had clinically overt vascular disease, which

may have obscured subtle oxidative stress differences related to kidney function. Moreover, circulating F2-isoprostane levels may not be representative of the local tissue oxidant/anti-oxidant ratio.
Another limitation, particularly of the clinical studies in this work (**Chapters 3** and **5.2**), is related to statistical power issues. Despite the successful inclusion of a large number of patients in the biopsy study protocol (**Chapter 3**), the number of artery biopsies of sufficient quality for interpretation was rather limited. Likewise, the number of patients in the genotype association study (**Chapter 5.2**) may have limited the ability to differentiate the effects of the genotypes. Obviously in both studies, the subgroup analyses suffer most from suboptimal power. We feel however that the findings all point in a clear direction and therefore believe that the power issue most probably results in underestimation rather than overestimation of the findings. We acknowledge that the use of a semiquantitative scoring system for the immunohistochemical stainings and the complexity of the lesions in the biopsy study (**Chapter 3**) further contribute to the power issue mentioned above. Nevertheless, the reliance on *in vivo* material from well-characterized patient groups, along with the availability of reliable, high quality extracted mRNA should be regarded as strengths of this study.
A third issue worth mentioning is the fact that no direct biological endpoint of enzymatic HO-1 activity was measured in our patients. While it is generally accepted and supported by luciferase promoter constructs and transient transfection assays in different cell lines that the studied polymorphisms affect HO-1 enzymatic activity, a biological *in vivo* assay of enzymatic activity would have strengthened our results. The same criticism may apply to the *in vitro* study (**Chapter 4**). However, the effect generated by the Hemin-induced overexpression of HO-1 suggests intact HO-1 activity.
Finally, determination of the patient characteristics in the clinical studies (**Chapters 3** and **5.2**) was based on retrospective file review. The review process was rigorously performed by two investigators who were blinded for the specific study variables. All available file data and investigations for documentation of CVD were taken into account as mentioned in the respective methods sections. Still, residual bias cannot be completely excluded. Furthermore, the studied populations are selected subgroups of CKD5D patients. Indeed, the study reported in **Chapter 3** focuses on patients with established and clinically relevant cardiovascular disease and the patients described in **Chapter 5.2** are younger with lower comorbidity scores than the average patients in our and many other Western European dialysis centers. Further investigation is warranted in both predialysis CKD patients as well as in non-selected CKD5D patients.

Conclusion

In conclusion, the present project adds to the current understanding of the accelerated atherosclerosis process seen in CKD. From the findings, it is clear that the uremic

environment increases the vulnerability to oxidative stressors of the vasculature as a whole as well as of the endothelial cells in particular. Heme oxygenase-1 was studied in many different conditions and its expression appears not to be impacted by CKD at first sight. However, taking the stress-responsive nature of the enzyme into account, the findings of both our clinical and *in* vitro studies may suggest relative deficiency of the HO-1 defense mechanism in uremia. Irrespective of the above interpretation, we found that known functional HO-1 promoter polymorphisms play a similar role in CVD in CKD patients as they do in the general population. Furthermore an interventional strategy, consisting of strong selective induction of HO-1 was capable of restoring the uremia-induced cell vulnerability. Given the pluripotency of HO-1 with its many anti-inflammatory and diverse anti-oxidative effects we believe that HO-1 constitutes an attractive and promising target molecule for further interventional research. The exact protective mechanism of HO-1 overexpression in this setting needs further elucidation.

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Chapter 7

Future prospects

Future Prospects

I. Completing the study of cell types involved in atherosclerosis: *in vitro* study of monocytes and vascular smooth muscle cells (VSMC)

In this dissertation, HO-1 expression was studied *in vivo* in human atherosclerotic plaques and *in vitro* in human umbilical artery endothelial cells (HUAECs). To further complete the evaluation of HO-1 in uremia related atherosclerosis, next steps will include the study of HO-1 expression in monocytes and VSMC in uremic and healthy conditions as well as the effect of HO-1 overexpression on the uremia-induced changes in these cells. Human peripheral mononuclear cells isolated from dialysis patients, compared to those of healthy volunteers, will be of particular interest, since these cells will reflect real-life uremic and healthy preconditioning, respectively.

II. In-depth study of the pathways that precede and follow HO-1 induction

HO-1 induction may result from a variety of stimuli that initiate diverse intracellular transcription regulation processes. Moreover, the enzyme instigates a broad range of anti-inflammatory, anti-apoptotic and anti-oxidative effects. To study the pathways that precede and follow HO-1 induction, gene expression studies using micro-array analysis will be performed.

In a first phase we will focus on a set of specific pre-defined pathways: genes related to the generation of ROS, signaling cascades and redox-dependent transcription factors, other anti-oxidative enzyme systems that are activated through the same ARE system as HO-1 and genes involved in the anti-inflammatory and anti-apoptotic functions of HO-1 will be evaluated. Special attention will be paid to the HO-1 regulating transcription factors Keap1/Nrf2 system and the transcription repressor Bach1[1]. In a second phase, we aim at discovering broader molecular interaction networks that may be affected differentially in uremic vs. healthy conditions. The analyses may shed light on how oxidative stress and inflammation pathways affect other pathways relevant to the atherosclerosis process.

a. µ-array analysis of the effect of oxidative stress on endothelial cell toxicity in uremia

RNA from SIN-1 treated HUAECs grown in uremic and healthy serum conditions have already been subjected to the micro-array methodology. Labeled cRNA samples were hybridized on Agilent G4112F full Human Genome 4x44K arrays using the Agilent protocols for gene expression analysis. Microarrays were scanned on an Agilent DNA

microarray scanner (G2565BA) and further processed using Agilent Feature Extraction Software. Statistical analysis of the data and validation studies are still to be carried out. Differentially expressed genes will be confirmed using Taqman real-time PCR technology on a Lightcycler 480. (Collaboration with Unit of Environmental Risk and Health, VITO, P. De Boever).

b. µ-array analysis of atherosclerotic arterial tissue of CKD and nonCKD patients

A set of arterial biopsies from clinically and phenotypically matched CKD (n=10) and non-CKD patients (n=10) will be selected and RNA extraction performed according to the method described in Chapter 3.

Labeled cRNA samples will be hybridized on Agilent G4112F full Human Genome 4x44K arrays using the Agilent protocols for gene expression analysis. Microarrays will be scanned on an Agilent DNA microarray scanner (G2565BA) and further processed using Agilent Feature Extraction Software Genespring 12 for analysis and biological interpretation of the data. More advanced pathway analysis will be done using Ingenuity Pathway Analysis. Differentially expressed genes will be confirmed using Taqman real-time PCR technology on a Lightcycler 480. (Collaboration with Unit of Environmental Risk and Health, VITO, P. De Boever).

III. Further exploration of the role of HO-1 polymorphisms in CKD: atherosclerosis and beyond

a. HO-1 polymorphisms and cardiovascular disease in a non-dialyzed CKD G3a-5 population

A group of CKD and nonCKD patients planned for vascular intervention and a group of CKD patients at the day of pre-emptive kidney transplantation have consented to sample DNA for HO-1 genotyping. Distribution of genotypes within CVD and nonCVD subgroups of these non-dialyzed CKD G3a-5 and nonCKD patients will be evaluated, compared and mirrored to the published literature data of nonCKD patients according to the methodology described in Chapter 5.1.

b. HO-1 polymorphisms and peritoneal membrane characteristics in a PD population

Peritoneal dialysis (PD) is a renal replacement method in which toxic waste products, electrolytes and fluid are removed across the peritoneal membrane through equilibration with a dialysis fluid that is instilled in the abdomen. There is a wide inter-individual variation in the solute transport characteristics of the peritoneal membrane. Based on a so-called Peritoneal Equilibration Test (PET), patients are categorized into fast, average and slow transporters. Faster transporter status was found to be associated with worse technique and patient outcome. This is explained at least partly

by the rapid dissipation of the osmotic glucose gradient between instilled fluid and plasma in these patients, as a consequence of which fluid removal may be suboptimal. On the other hand, fast transporter status has also been linked to local and systemic micro-inflammation and oxidative stress. Since HO-1 is also expressed in the peritoneum, we hypothesize that HO-1 promoter polymorphisms are associated with baseline small solute peritoneal transport assessed by PET in PD patients. At present, data of a retrospective study of 167 PD patients treated between 2002 and 2013 at three Belgian hospitals (University Hospitals Leuven, n=123; ZNA Stuivenberg Antwerp, n=30; Jessa Hasselt, n=14) are available. Baseline demographic, clinical, biochemical and PET data ($D/P_{creatinine}$ and $D/D0_{glucose}$) were retrieved from the patient files. Stored whole blood samples were used for DNA extraction and HO-1 genotyping. (GT)n dinucleotide repeat length was determined by fragment analysis on the ABI3730 sequence platform. Long (L) and short (S) alleles were defined as ≥25 and <25 repeats based on literature data. The A(-453)T SNP was detected by a Taqman assay. In a preliminarly analysis, thirty-five percent of the patients expressed at least one short (GT)n repeat allele AND one A SNP allele. This combination of genotypes (Group A) can be considered as having a higher HO-1 activity as compared to the remainder of the patients (Group B). Comparison of these two patient groups revealed statistically significant differences in $D/P_{creatinine}$, $D/D0_{glucose}$, body mass index (BMI), serum albumin, total cholesterol and C-reactive protein (CRP) levels. The findings are supportive for a role of promotor polymorphisms of HO-1 in the variability of peritoneal small solute transport characteristics, as patients with higher HO-1 expression have slower peritoneal small solute transport rates and lower levels of inflammatory markers. Further analysis of the data will be performed.

IV. Interventional studies aiming at amelioration of the uremic phenotype

a. Improving the antioxidant / oxidant balance

As mentioned in the introduction, diverse mechanisms in CKD, both endogenous and exogenous, lead to increased activity of oxidative enzymes such as NOX, MPO and XO, the dysregulation of crucial enzymes such as the phenomenon of eNOS uncoupling or the accumulation of secondary radicals and transition metals. In addition many crucial antioxidative mechanisms have been found to be impaired. Moreover, many of these protective mechanisms at least interact with Heme Oxygenase-1. A promising field of renewed interest which might be further explored in clinical studies in CKD patients

comprises thus interventions for simple dietary deficiencies and newly discovered compounds such as trace elements, flavonols and tempol.

b. Targeted HO-1 induction

When the beneficial effect of HO-1 induction is confirmed in additional studies and experiments on peripheral blood monocytes, strategies for HO-1 overexpression and its effects on the uremic phenotype will be further investigated. Previously, animal models with either hemin-induced HO-1 overexpression [2, 3] or adenoviral vector mediated HO-1 gene delivery[4, 5] resulted in a clear reduction of plaque formation and increase in plaque stability. Therefore, in a APO E $^{-}/^{-}$ mouse model, CKD can be induced by 5/6th nephrectomy and the effect of HO-1 overexpression on the generation of atherosclerosis and oxidative stress could be investigated in the CKD versus control APO E$^{-}/^{-}$ mice groups by either hemin or gene therapy.

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Chapter 8

Summary / Samenvatting

Summary

In advanced kidney disease the prevalence of atherosclerotic cardiovascular disease is high and nearly equivalent across age groups. Recent data indicate that even minor renal dysfunction impacts on the onset of cardiovascular disease (CVD). Many authors hypothesize that increased oxidative stress is the unifying concept that initiates and feeds this problem. Several findings in uremic patients point towards an imbalance favoring the pro-oxidative state: uremia-related as well as dialysis-related factors have been described to lead to the increase of oxidants and decrease of the antioxidative capacity.

In **Chapter 1** we summarized current knowledge on pathophysiological mechanisms of oxidative stress, antioxidant defense mechanisms and atherosclerotic vascular disease in uremia. Diverse mechanisms, both endogenous as exogenous, lead to the increased activity of oxidative enzymes, the accumulation of secondary radicals and/or transition metals and the impairment of crucial antioxidative mechanisms.

The overall aim of this thesis was to explore the anti-oxidative and anti-atherosclerotic enzyme Heme oxygenase-1 (HO-1) in the context of uremia and accelerated atherosclerosis (**Chapter 2**).

In **Chapter 3** we report on our evaluation of atherosclerotic plaque morphology and HO-1 expression in patients with known advanced atherosclerotic disease of the peripheral arterial vasculature. In 66 biopsies of arteries procured during clinically indicated vascular surgery procedures, atherosclerotic lesions in CKD patients were characterized by higher inflammatory cell infiltration, more neovascularization and a higher proportion of plaque complications than seen in lesions of patients without renal insufficiency. In agreement, the degree of renal impairment (eGFR) was found to be an important predictor of both intra-plaque neovascularization and atherosclerotic plaque complications, independent of traditional cardiovascular risk factors. Atherosclerotic lesions in CKD patients had a tendency to lower expression of the anti-atherogenic enzyme HO-1.

To further explore the effect of the uremic environment on HO-1 expression in the early phase of atherogenesis, we conducted an *in vitro* assay in human umbilical arterial endothelial cells (HUAECs) preconditioned with either healthy or uremic human serum, as described in **Chapter 4**. Moreover, we studied the effect of HO-1 overexpression on oxidative stress-induced cell injury in both healthy and uremic conditions. We found that HUAECs show a higher vulnerability to the oxidative stressor $ONOO^-$ after preconditioning in uremic serum as compared to healthy serum. This could not be explained by differential effects of the culture conditions on the expression of the protective enzyme HO-1 or reactive oxygen species (ROS) generation. The exact mechanism behind the increased vulnerability remains to be unraveled. Interestingly however, Hemin-induced HO-1 overexpression mitigated this cell vulnerability in

uremic conditions by normalizing the apoptotic tendency and by reducing cytoplasmic ROS generation.

In **Chapter 5** two studies on the impact of known functional HO-1 promoter polymorphisms on the occurrence of CVD are reported. In **Chapter 5.1**, we describe the results of a systematic review of the literature on the role of the HO-1 (GT)n repeat polymorphism in the development of atherosclerotic disease in the general population. Shorter repeats with (GT)n<25 are associated with higher inducibility and activity of HO-1. The epidemiological association between HO-1 (GT)n promoter polymorphism and CVD seen in our review, confirmed the presumed protective effects of HO-1 enzymatic activity. In addition, variability in genotype distribution between ethnic groups was shown, which should be taken into account when interpreting data from published studies.

In **Chapter 5.2** we studied the role of the HO-1 (GT)n repeat polymorphism and the HO-1 T(-413)A Single Nucleotide Polymorphism (SNP) in the occurrence of CVD in a dialysis population. Like shorter (GT)n repeats, the A allele of the SNP is associated with higher inducibility and activity of HO-1. Our study provided evidence for a role of the (GT)n repeat promoter polymorphism of the HO-1 enzyme in the occurrence of CVD in a dialysis population. Dialysis patients with a biallelic long genotype (lower HO-1 expression) had an increased risk for CVD compared to patients with biallelic short genotype (higher HO-1 expression). On the other hand we could not show any significant association between the T(-413)A SNP and CVD.

From a detailed discussion of our experimental data (**Chapter 6**), we conclude that the present project adds to the current understanding of the accelerated atherosclerosis process seen in CKD. From our findings, it is clear that the uremic environment increases the vulnerability to oxidative stressors of the vasculature as a whole as well as of the endothelial cells in particular. Heme oxygenase-1 was studied in many different conditions and its expression appears not to be impacted by CKD at first sight. However, taking the stress-responsive nature of the enzyme into account, the findings of both our clinical and *in* vitro studies may suggest relative deficiency of the HO-1 defense mechanism in uremia. Irrespective of the above interpretation, we found that known functional HO-1 promoter polymorphisms play a similar role in CVD in CKD patients as they do in the general population. Furthermore an interventional strategy, consisting of strong selective induction of HO-1 was capable of restoring the uremia-induced cell vulnerability. Given the pluripotency of HO-1 with its many anti-inflammatory and diverse anti-oxidative effects we believe that HO-1 constitutes an attractive and promising target molecule for further interventional research. The exact protective mechanism of HO-1 overexpression in this setting needs further elucidation.

Samenvatting

Bij gevorderd nierfalen is het voorkomen van hart- en vaatziekten ten gevolge van atherosclerose (of aderverkalking) hoog en vergelijkbaar tussen verschillende leeftijdscategorieën. Recent onderzoek toonde aan dat er al bij milde nierfunctie-stoornissen een impact is op het ontstaan van hart- en vaatziekten. Meerdere auteurs stelden de hypothese dat “oxidatieve stress” het verbindende mechanisme is wat hart- en vaatziekten initieert en voedt bij patiënten met nierfalen. Meerdere bevindingen bij uremische patiënten (patiënten met onvoldoende door de nieren geklaarde afvalstoffen in het bloed) wijzen in de richting van toegenomen aanwezigheid van oxidantia. Zoals reeds eerder beschreven in de literatuur, kunnen zowel uremie gerelateerde als dialyse gerelateerde factoren leiden tot een toename van oxidantia en een afname van anti-oxidatieve capaciteit.

H**oofdstuk 1** is een samenvatting van de huidige kennis van alle pathofysiologische mechanismen ten aanzien van oxidatieve stress, anti-oxidatieve mechanismen en aderverkalking gerelateerde hart- en vaatziekten in uremie. Diverse mechanismen, zowel endogeen (vanuit het lichaam zelf) als exogeen (door beinvloeding van buitenaf), leiden tot een toegenomen activiteit van oxidatieve enzymes, accumulatie van (secundaire) radicalen en/of transitiemetalen, en een verminderd functioneren van diverse cruciale anti-oxidatieve mechanismen.

De algehele doelstelling van deze thesis was het bestuderen van het anti-oxidatieve en anti-atherosclerotische enzyme Heem oxygenase-1 (HO-1) in de context van uremie en versnelde atherosclerose (**hoofdstuk 2**).

In **hoofdstuk 3** rapporteren we onze evaluatie van atherosclerotische plaque morfologie en HO-1 expressie bij patiënten met gekende gevorderde atherosclerotische ziekte van het perifere vaatbed. In 66 biopsies van slagaders die verzameld werden bij klinisch geïndiceerde vasculaire chirurgie, toonden we aan dat atherosclerotische letsels bij nierpatiënten gekenmerkt waren door meer infiltratie van ontstekingscellen, meer nieuwvorming van bloedvaten, en een hogere proportie van plaques complicaties, in vergelijking met controles zonder nierinsufficiëntie. De mate van nierfunctiebeperking was bovendien een belangrijke voorspeller van zowel nieuwvorming van bloedvaten in de plaque als van het voorkomen van plaque complicaties, onafhankelijk van traditionele risicofactoren voor hart- en vaatziekten. Bloedvatletsels bij nierpatiënten hadden een tendens om in mindere mate het anti-atherogene enzyme HO-1 tot expressie te brengen.
Om het effect van een uremische omgeving op de HO-1 expressie in een vroege fase van de atherogenese te bestuderen voerden we een in-vitro studie uit, beschreven in **hoofdstuk 4**. Deze studie bestudeert het gedrag van endotheelcellen (humane

navelstreng arteriële endotheelcellen (HUAECs)) blootgesteld aan oxidatieve stress, enerzijds in een omgeving van serum van gezonde personen, en anderzijds in een omgeving van serum van personen met uremie. Bijkomend bestudeerden we het effect van HO-1 overexpressie op celbeschadiging veroorzaakt door oxidatieve stress, opnieuw in een milieu van zowel gezond als uremisch serum.
We stelden vast dat endotheelcellen (HUAECs) een hogere kwetsbaarheid vertoonden voor het oxidant $ONOO^-$ in een uremisch milieu in vergelijking met een gezond milieu. Dit kan niet verklaard worden door de uiteenlopende effecten van kweekcondities op de expressie van het beschermende enzyme HO-1 of de generatie van ROS. Het exacte mechanisme achter de toegenomen kwetsbaarheid is nog niet ontrafeld. Intrigerend is dat Hemin-geïnduceerde HO-1 overexpressie deze kwetsbaarheid van de cel herstelt in uremische condities doordat de neiging tot apoptose normaliseert en de cytoplasmatische generatie van ROS vermindert.
Hoofdstuk 5 betreft twee studies over de impact van gekende functionele HO-1 promotor polymorfismen op het voorkomen van hart- en vaatziekten. In **hoofdstuk 5.1** beschrijven we de resultaten van een systematisch nazicht van de bestaande literatuur over de rol van de HO-1 $(GT)_n$ herhalings polymorfismen in het onstaan van atherosclerotische ziekte in de algehele populatie. Kortere herhalingen met $(GT)_n$ <25 zijn geassocieerd met een hogere induceerbaarheid en activiteit van HO-1. Deze epidemiologische associatie tussen HO-1 $(GT)_n$ promotor polymorfisme en hart- en vaatziekte vastgesteld in onze literatuurstudie, bevestigde de veronderstelde beschermende effecten van HO-1 enzymatische activiteit. De data wijzen ook uit dat er belangrijke variatie is in genotype distributie tussen etnische groepen die belangrijk zijn voor interpretatie van gepubliceerde studieresultaten.
In **hoofdstuk 5.2** bestudeerden we de rol van HO-1 $(GT)_n$ herhalings polymorfisme en van de HO-1 T(-413)A Single Nucleotide Polymorphism (SNP), in het voorkomen van hart- en vaatziekte in een dialyse populatie. Zoals kortere $(GT)_n$ herhalingen, is het A allel van het SNP geassocieerd met een hogere induceerbaarheid en activiteit van HO-1. Onze studie levert evidentie voor een rol van $(GT)_n$ herhalings promotor polymorfisme van het HO-1 enzyme in het voorkomen van hart- en vaatziekten in een dialyse populatie. Dialyse patiënten met het bi-allelisch lang genotype dat gepaard gaat met een lagere HO-1 expressie, hadden een toegenomen risico voor hart-en vaatziekten, in vergelijking met het bi-allelisch kort genotype dat gepaard gaat met een hogere HO-1 expressie. We konden evenwel geen associatie aantonen tussen de T(-413)A SNP en het voorkomen van hart- en vaatziekten.
Uit een gedetailleerde bespreking van onze experimentele data (**hoofdstuk 6**), kunnen we concluderen dat het huidige project bijdraagt aan de huidige kennis van het versnelde atherosclerotische proces bij chronische nierinsufficiëntie. Uit onze bevindingen blijkt dat de uremische omgeving de kwetsbaarheid voor oxidatieve stress verhoogt; In het arteriële vaatbed als geheel zowel als in de endotheelcellen in het bijzonder. Heem oxygenase-1 werd onderzocht in een groot aantal verschillende

omstandigheden en op het eerste gezicht lijkt de expressie van het enzym niet beïnvloed te worden door nierfalen. Rekening houdend met de aard van het enzyme om op stress te reageren, suggereren de resultaten van zowel onze klinische als in vitro studies een relatieve deficiëntie van het HO-1 verdedigingsmechanisme bij uremie. Ongeacht de bovenstaande interpretatie, vonden we dat gekende functionele HO-1 promotor polymorfismen een vergelijkbare rol spelen bij hart- en vaatziekten in nierpatiënten als in de algemene bevolking. Een interventie-strategie, die bestaat uit een sterke selectieve inductie van HO-1 kon de door uremie geïnduceerde kwetsbaarheid van de cel herstellen. Gezien de veelzijdigheid van HO-1 met zijn vele anti-inflammatoire en diverse anti-oxidatieve effecten, geloven wij dat HO-1 een aantrekkelijk en veelbelovend doelmolecule vormt voor verder interventioneel onderzoek. Het exacte beschermende mechanisme van HO-1 overexpressie in deze context dient verder uitgezocht te worden.

Curriculum vitae

Curriculum vitae

Personal information

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Publications

Publications

Evenepoel P, **Daenen K**, Bammens B, Claes K, Meijers B, Naesens M, Sprangers B, Kuypers D, Lerut E. Microscopic nephrocalcinosis in chronic kidney disease patients. Nephrol Dial Transplant. 2015;30(5):843-8.

Daenen K, Martens P, Bammens B. Association of HO-1 (GT)n promotor polymorphism and cardiovascular disease: a reanalysis of the literature. Can J Cardiol. 2016;32(2): 160-8.

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Daenen K, Verbeken E, Fourneau I, Hoylaerts M, Kuypers D, Bammens B. Atherosclerotic lesion characterization in CKD : The 'vulnerable plaque' phenotype.

Daenen K, Hoylaerts M, Kuypers D, Bammens B. Heme oxygenase-1 overexpression mitigates oxidative stress induced apoptosis in uremic endothelial cells.

Abstract

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Daenen K, et al. Atherosclerotic plaque characterization and heme oxygenase-1 expression in chronic kidney disease: the 'vulnerable plaque phenotype. ERA-EDTA. Istanbul, Turkey 18-21/05/2013

Daenen K, et al. Heme oxygenase-1 expression by human umbilical artery endothelial cells (huaecs) in uremic versus healthy serum conditions. ERA-EDTA. Istanbul, Turkey 18-21/05/2013

Daenen K, et al. Heme oxygenase-1 (HO-1) (gt)n repeat promotor polymorphism distribution in peripheral artery disease (pad) patients: is the protective effect of ho-1 against atherosclerosis overruled by uremia. ERA-EDTA. Istanbul, Turkey 18-21/05/2013

Daenen K, et al. Atherosclerotic plaque characterization and HO-1 expression in Chronic Kidney Disease: The 'vulnerable plaque phenotype'. ASN. Atlanta, USA 9/11/2013

Daenen K, et al. Heme oxygenase-1 expression BY HUAEC in uremic versus healthy serum conditions. ASN. Atlanta, USA 9/11/2013

Daenen K, et al. Heme oxygenase-1 Polymorphisms and Peritoneal Membrane Characteristics in Peritoneal Dialysis Patients. ASN. Philadelphia 11/2014

Daenen K, et al. Atherosclerotic Plaque Characterization in Chronic Kidney Disease (CKD). ASN. Philadelphia 11/2014

Abstracts for Oral Presentation

Daenen K, et al. Heme oxygenase-1 (HO-1) (GT)n repeat promotor polymorphism distribution in peripheral artery disease (PAD) patients: is the protective effect of HO-1 against atherosclerosis overruled by uremia. BVN-SBN. Musée de la Médecine, Bruxelles 25-4-2013

Daenen K, et al. Heme oxygenase-1 expression by human umbilical artery endothelial cells (huaecs) in uremic versus healthy serum conditions. BVN-SBN. Musée de la Médecine, Bruxelles 25-4-2013

Daenen K, et al. Atherosclerotic plaque characterization and heme oxygenase-1 expression in chronic kidney disease: the 'vulnerable plaque phenotype. BVN-SBN. Musée de la Médecine, Bruxelles 25-4-2013

Daenen K, et al. Heme oxygenase-1 expression BY HUAEC in uremic versus healthy serum conditions. ISBP. Bologna, Italy 13/9/2013

Daenen K, et al. HO-1 (GT)n repeat promotor polymorphism in PAD patients: is the protective effect of HO-1 overruled by uremia? ISBP. Bologna, Italy 13/9/2013

Daenen K, et al. Atherosclerotic plaque characterization and heme oxygenase-1 expression in Chronic Kidney Disease: The 'vulnerable plaque phenotype'. ISBP. Bologna, Italy 13/9/2013

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Awards

4-2013 National Grant for Belgium for outstanding scientific presentation annual meeting of the Belgian Society of Nephrology, Brussels

10-2014 Best abstract and presentation Annual meeting of the Belgian Society of Nephrology, Brussels